Mast cell activation and mediator release : implications for the cardiovascular system

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**MAST CELL ACTIVATION AND MEDIATOR
RELEASE: IMPLICATIONS FOR THE
CARDIOVASCULAR SYSTEM**

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MAST CELL ACTIVATION AND MEDIATOR RELEASE: IMPLICATIONS FOR THE CARDIOVASCULAR SYSTEM

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door

Charles Marie Catharine Joseph van Haaster

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Promotores:

Prof. dr. G.J. Van der Vusse

Prof. dr. G. Hornstra

Co-promotor:

Dr. W. Engels

Beoordelingscommissie:

Prof. dr. ir. W.H.M. Saris (voorzitter)

Prof. dr. M. Borgers

Dr. J.W.M. Heemskerk

Prof. dr. A. van der Laarse (Rijksuniversiteit Leiden)

Prof. dr. R.S. Reneman

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Ik...

**Ik ben een piepklein stukje
van het wonder...
Maar boven mijn pet gaat zoveel
meer dan er onder**

(Toon Hermans)

Aan mijn ouders

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ABBREVIATIONS

16:0	palmitic acid
18:0	stearic acid
16:1(n-7)	palmitoleic acid
18:1(n-9)	oleic acid
18:2(n-6)	linoleic acid
18:3(n-3)	α -linolenic acid
18:3(n-6)	γ -linolenic acid
18:4(n-3)	stearidonic acid
20:3(n-6)	dihomo- γ -linolenic acid
20:3(n-9)	Mead acid
20:4(n-6)	arachidonic acid
20:5(n-3)	timnodonic acid, eicosapentaenoic acid
22:3(n-9)	dihomo-mead acid
22:4(n-6)	adrenic acid
22:5(n-6)	Osmond acid
22:5(n-3)	clupanodonic acid
22:6(n-3)	cervonic acid, docosahexaenoic acid
AA	arachidonic acid
$[Ca^{2+}]_i$	cytosolic free Ca^{2+} concentration
BHT	3,5-di- <i>tert</i> -butyl-4-hydroxytoluene
CF	coronary flow
CFLC	cardiac fibroblast-like cells
CK	creatine kinase (EC 2.7.3.2)
CMC	cardiomyocytes
CO	cyclooxygenase
CTMC	connective tissue mast cell
DAB	diaminobenzidine
DAG	diacylglycerol
DHA	docosahexaenoic acid, cervonic acid
DHC	methyl-2,5-dihydroxycinnamate
DMSO	dimethyl sulfoxide
EC	endothelial cells
EPA	eicosapentaenoic acid, timnodonic acid
ER	endoplasmic reticulum
F_{ceRI}	immunoglobulin E receptor
FCS	foetal calf serum
G protein	GTP-binding protein
G_{i3}	specific GTP-binding protein
GS	genistein
HCO	hydrogenated coconut oil
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HETE	hydroxyeicosatetraenoic acid

5-HT	5-hydroxytryptamine, serotonin
HHT	hydroxyheptadecatrienoic acid
HODE	hydroxyoctadecadienoic acid
HPETE	hydroperoxyeicosatetraenoic acid
HPLC	high-performance liquid chromatography
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule-1
IgE	immunoglobulin E
IL	interleukin
Ins(1,4,5)P ₃	inositol (1,4,5) trisphosphate
LA	linoleic acid
LDH	lactate dehydrogenase (EC 1.1.1.27)
LPS	lipopolysaccharide
LTC ₄	leukotriene C ₄
LVDP	left ventricular developed pressure
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
MMC	mucosal mast cell
MO	mackerel oil
MUFA	monounsaturated fatty acids
NO	nitric oxide
OPA	o-phthalaldehyde
PAF	platelet-activating factor
PC 19:0	1,2 dinonadecanoyl-sn-glycero-3-phosphocholine
PG	prostaglandin
PGDS	PGD ₂ synthase
PGI ₂	prostacyclin
PKC	protein kinase C
PLA ₂	phospholipase A ₂
PLC	phospholipase C
PO	peroxidase (EC 1.11.1.7)
PUFA	polyunsaturated fatty acid
RHEC	rat heart endothelial cell
RMCP I	rat mast cell proteinase I
ROC	receptor-operated channel
SAFA	saturated fatty acids
SCF	stem cell factor
SO	sunflowerseed oil
SRC	store-regulated channel
TxB ₂	thromboxane B ₂
TyrK	protein tyrosine kinase(s)
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
VIP	vasoactive intestinal peptide

CHAPTER 1

Introduction and Objectives of the Thesis

1.1 History and Characteristics of Mast Cells

In his doctoral thesis²⁴ (1878), Paul Ehrlich described the presence of cells residing in connective tissue near blood vessels, which contained prominent cytoplasmic granules with a high affinity for certain basic dyes. These cells were considered to be 'overfed' and were, therefore, called 'mast cells', derived from the German word 'Mastung'. Early indications that these cells were involved in hypersensitivity reactions came from Unna⁹⁹, who reported many of these cells in the cutaneous lesions of patients with *urticaria pigmentosa*. A hypersensitivity reaction was firstly described by Portier *et al.*⁸⁰ in dogs given sea anemone toxin. Subsequently, it was shown that serum from affected animals could be used to transfer this hypersensitivity reaction to non-affected animals.⁴ In 1921 Prausnitz *et al.*⁸¹ demonstrated that serum from a person allergic to horses, when injected into human skin, induced a hypersensitivity reaction when the recipient was challenged with antigen to which the original donor was sensitive. The serum 'reagins' have later been identified as a specific immunoglobulin class termed immunoglobulin E (IgE), the cells in the skin sensitized by IgE were identified as mast cells. Since that time, mast cells have been shown to express specific membrane receptors ($F_{c\epsilon}R1$) that specifically bind the F_c portion of IgE antibodies with high affinity.¹³ The cross-linkage of two IgE molecules by allergens triggers the mast cell to undergo an integrated, noncytolytic series of biochemical and ultrastructural alterations, commonly known as exocytosis, resulting in the exposure of the matrices of the cytoplasmic granules to the external environment and release of compounds such as histamine and proteases, stored in the granules. This is associated with *de novo* synthesis and release of other mediators such as prostaglandins and/or leukotrienes, compounds which are responsible for some of the clinical symptoms associated with immediate hypersensitivity.^{30,93}

In contrast to their circulating counterpart, the basophil, mast cells are absent in the blood and are ordinarily distributed throughout normal connective tissue and mucosal surfaces. Mast cells are often found in the close vicinity of blood vessels and beneath epithelial surfaces, such as those of the respiratory and gastrointestinal systems and the skin, areas that are frequently exposed to environmental antigens.⁹³ Mast cells have also been found in various tissues and organs such as the peritoneum, skin, lung, gut, stomach, thymus, liver and the heart.^{30,32}

1.2 Mast Cell Heterogeneity, Distribution and Origin

Mast cells from different anatomical locations exhibit readily discernible differences in morphology and granule content.^{6,25} Due to their localization and histochemical staining properties, the cells are subdivided into *connective tissue-type* mast cells (CTMC), present in skin, peritoneal cavity, heart and at numerous other locations, and *mucosal* mast cells (MMC), present in large numbers in the mucosa of the midgut and the lung. Basic differences between these two mast cell populations are summarized in Table 1.1 and are discussed below.

Table 1.1: *Major Differences between Mast Cell Types*

	Mucosal Mast Cell (MMC)	Connective Tissue Mast cell (CTMC)
location <i>in vivo</i>	gut and lung	ubiquitous (peritoneum, skin, heart)
size	8-15 μm	9-20 μm
nucleus	unilobular or bilobular	unilobular
granules	small and few	large and many
life span	<40 days	>40 days
major AA metabolite	LTC ₄	PGD ₂
ratio LTC ₄ /PGD ₂	25:1	1:40
histamine/serotonin content	+	++
toluidine blue staining at neutral pH	-	+
major proteoglycan	chondroitin sulphate E	heparin
T-cell dependent proliferation	+	-
mediators important in proliferation	interleukins	stem cell factor

Adapted and modified from Roitt⁸⁵

The mature CTMC is a relatively large cell with a single, small ovoid nucleus and a cytoplasm packed with numerous, uniformly dense, membrane-bound granules (Fig. 1.1).²³ These cells are relatively long-lived (>40 days) and contain the usual array of subcellular organelles, including mitochondria, ribosome-bound endoplasmic reticulum, a Golgi complex and abundant submembranous filaments. CTMC may contain up to 1000 small secretory granules (0.2 to 0.4 μm in diameter), which occupy about 50-55% of the total cytoplasmic volume. In contrast, MMC are somewhat smaller than their connective tissue counterparts and their granules are fewer in number. MMC have a lower histamine and serotonin content than CTMC, which is most probably due to a reduced number of granules.²⁵ Major arachidonic acid (AA) metabolites (prostaglandins and leukotrienes) are produced by both mast cell types, but in different amounts. For example, the ratios of production of leukotriene C₄ (LTC₄) to prostaglandin D₂ (PGD₂) is 25:1 in the MMC and 1:40 in the CTMC, respectively.⁸⁶

CTMC are often indicated as 'typical' mast cells since they stain easily with various metachromatic dyes. For instance, CTMC usually stain with toluidine blue at neutral pH, whereas MMC stain poorly under these conditions and require acidic conditions to become stained.⁵ The good staining properties at neutral pH of CTMC probably results from the presence of large amounts of heparin within these cells.¹² MMC contain little or no heparin and, instead, comprise an oversulfated chondroitin, named chondroitin sulphate E.⁸³ Although both CTMC and MMC respond to immunological activation and to the calcium ionophore A23187, they differ in their response to various other agonists (Fig. 1.1). Thus, MMC, in contrast to CTMC, are totally unresponsive to the 'classical' mast cell-degranulating agent compound 48/80, somatostatin, bradykinin and also to the vasoactive intestinal peptide (VIP).

Figure 1.1: Mediators and agonists in different types of mast cells

Mast cells are progenies of multipotential hematopoietic stem cells.^{52,74} Most other progenies of these multipotential stem cells, such as erythrocytes, neutrophils and basophils, leave the bone marrow after they have differentiated. However, mast cells do not complete their differentiation in the hematopoietic tissue. Morphologically unidentified precursors leave the bone marrow, enter the blood and subsequently migrate into connective or mucosal tissue, where they proliferate and differentiate into morphologically identifiable CTMC or MMC due to various factors present in the microenvironment.⁵¹ Mast cell proliferation/differentiation has been shown to be mediated by T cell-dependent and fibroblast-dependent mechanisms.³ T cell-dependent mechanisms are predominantly responsible for the proliferation/differentiation of MMC from mast cell progenitors, a process which is stimulated by interleukins (IL) such as IL-3, IL-4, IL-9 or IL-10.^{35,46,58,96} In contrast, the proliferation/differentiation of mast cell progenitors to mature CTMC is largely dependent on stem cell factor (SCF), produced by fibroblasts.⁹⁷

1.3 Mediators Released from Activated Mast Cells

Mast cell granules contain a variety of mediators which are rapidly released upon activation. In addition, mast cells release a variety of *newly formed* mediators, such as eicosanoids, hydroxy fatty acids and leukotrienes. Various mast cell-derived mediators and their biological functions are summarized in Table 1.2. For the sake of clarity, only some aspects of mediators relevant to the thesis will be discussed.

1.3.1 Histamine

Histamine is the best-known mediator released from mast cells. In peritoneal mast cells, which belong to the connective tissue-type, histamine is present in high amounts and constitutes about 10% of the dry weight of these cells.⁵⁶ In tissues, histamine is predominantly stored in secretory granules of mast cells, where it binds by electrostatic interactions with the carboxyl groups of heparin.⁸⁴

Table 1.2: *Various Mast Cell-derived Mediators and their Biological Functions*

Mediator	Action	References
<i>Mediators in secretory granules</i>		
Chymase/ Proteinase	mucus secretion † smooth muscle contraction † degradation of neuropeptides	20,78
Histamine	vasodilation positive chronotropic/inotropic effects on the heart bronchoconstriction endothelial cell permeability † endothelial cell proliferation † gastric acid secretion †	41,76
5-HT	vasodilation vascular permeability †	28
<i>Newly formed mediators</i>		
LTC ₄	vasoconstriction mucus secretion † smooth muscle contraction †	78,102
LTB ₄	vascular permeability † neutrophil/eosinophil activation	14
NO	vasodilation platelet aggregation †	91
PAF	vascular permeability † leukocyte activation	15,42
PGD ₂	modification of local neurotransmission histamine-induced increase of capillary permeability † vasodilation	69,98
TNFα	vasoconstriction neutrophil/eosinophil activation bronchoconstriction	7,65,104

The biological effects of histamine in various cells (*see below*) are mediated through distinct receptors of at least three subtypes (H₁, H₂ and H₃). The signal transduction mechanisms by which histamine exerts its effects are different in each subtype. For instance, H₁-receptors are coupled to phosphoinositide-breakdown and Ca²⁺ mobilization, while H₂-receptors are linked to adenylate cyclase.⁷⁷ The precise mechanism involved in stimulus-response coupling following H₃-receptor activation has not yet definitively been clarified. Responses mediated through H₁-receptors are antagonized by antihistamines such as mepyramine, whereas those responses mediated

through H₂-receptors and H₃-receptors are antagonized by compounds such as cimetidine and thioperamide, respectively.^{41,77} Histamine evokes a wide range of biological effects which are similar for many animal species. However, large differences in sensitivity to this amine are observed between various species. For instance, in the rat, relatively high concentrations of histamine are required to evoke a vasodilatory effect, primarily mediated by H₁-receptors, whereas the guinea pig vasculature already responds to much lower histamine concentrations. Together with other factors released from activated mast cells, such as serine proteinases, lysosomal enzymes and tumor necrosis factor (TNF α), histamine contributes to the pathogenesis of inflammatory reactions, for instance by increasing vascular permeability.⁶⁴ Histamine has also been shown to be involved in gastric acid secretion, a process which is potently inhibited by histamine H₂-receptor antagonists.¹¹ In the lungs, histamine causes bronchoconstriction and therefore this compound plays an important role in asthma. Histamine has also been shown to enhance vascularization and endothelial cell proliferation, which implies the involvement of mast cells in the process of wound healing and tissue remodeling.^{29,76} Finally, histamine has positive inotropic as well as chronotropic effects on the heart and, induces a variety of arrhythmogenic responses in various species.⁶³

1.3.2 Membrane Fatty acid Composition and Release of Prostanoids: Role of the Diet

The fatty acid composition of membranes is a result of cellular *de novo* fatty acid synthesis and incorporation of fatty acids derived from the diet.⁴⁴ *De novo* synthesis of long-chain (poly-)unsaturated fatty acids starts from two precursor fatty acids, palmitic acid (16:0) and stearic acid (18:0), which may endogenously be formed by elongation of acetyl CoA (Fig. 1.2). Since palmitic acid and stearic acid need not necessarily be provided by the diet, these fatty acids are 'non-essential'. Both fatty acids are substrates for $\Delta 9$ desaturase, which introduces a double bond between the 9th and 10th carbon-atom (counted from the *carboxyl* group) into saturated fatty acids. $\Delta 9$ Desaturation of 16:0 results in the formation of 16:1(n-7), whereas similar action on 18:0 results in the formation of 18:1(n-9). In this notation, '18' denotes the total number of carbon-atoms, '1' the number of double bonds and '(n-7)' the position of the first carbon-atom involved in a double bond (counted from the terminal *methyl* group). Subsequent chain-elongation and $\Delta 6$ and/or $\Delta 5$ desaturation result in the formation of a variety of long-chain monounsaturated and polyunsaturated fatty acids of the (n-7) or (n-9) family, respectively. Since vertebrate cells lack $\Delta 12$ and $\Delta 15$ desaturases, they cannot *de novo* synthesize linoleic acid, 18:2(n-6), and α -linolenic acid, 18:3(n-3), the 'parent' fatty acids of the (n-6) and (n-3) family, respectively. These fatty acids, therefore, must be provided by the diet and are called 'essential fatty acids'. After $\Delta 6$ desaturation (Fig 1.2), subsequent chain-elongation and $\Delta 5$ -desaturation, linoleic acid, 18:2(n-6), is converted into arachidonic acid, 20:4(n-6), the predominant substrate for the eicosanoid synthesizing pathway (see below). Similar actions on α -linolenic acid, 18:3(n-3), results in the formation of timnodonic acid, 20:5(n-3). Fatty acids of the (n-3) and (n-6) family are known to compete with each other for desaturation by desaturase enzyme

systems.⁴⁴ In general, the affinity of substrate fatty acids for desaturation by a desaturase system increases with the number of double bonds. For instance, desaturation of linoleic acid by $\Delta 6$ desaturase is inhibited by α -linolenic acid and other long-chain polyunsaturated fatty acids.¹⁶

Figure 1.2: Major biosynthetic pathways of fatty acids

In plasma, fatty acids are transported by lipoproteins in the form of triacylglycerols or complexed to albumin. These fatty acids are liberated from triacylglycerols by the action of specific enzymes, such as lipoprotein lipases.³⁴ After transport through endothelial cells, fatty acids are presented to resident tissue mast cells. Once entered into these

cells, fatty acids are transported to the mitochondria where they are oxidized in peroxisomes or are stored in a triacylglycerol pool. In addition, fatty acids may be metabolized by intracellular enzyme systems or are incorporated in membrane phospholipids. When incorporated in phospholipids, polyunsaturated fatty acids are not only important constituents of cellular membranes, but, after liberation by the action of phospholipase A₂, they may also serve as precursor ('parent') fatty acids for the formation of prostanoids, hydroxy fatty acids and leukotrienes. Prostanoids comprise two different groups of compounds: prostaglandins and thromboxanes. All prostanoids contain 20 carbon-atoms arranged in a cyclic structure carrying two side-chains, one of which is terminated by a carboxyl group and the other by a methyl group. Depending on the configuration of the cyclic part of the molecule, prostanoids are divided into various 'families', indicated by the capitals A to I. Each prostanoid family consists of 1, 2 and 3-series, depending on the number of double bonds in their two side chains. Prostanoids from the 1 and 2-series are derived from dihomono- γ -linolenic acid, 20:3(n-6), and arachidonic acid, 20:4(n-6), respectively, whereas timnodonic acid, 20:5(n-3), is the substrate for the 3-series of prostanoids. In most animal cells, the arachidonic acid content is much higher than that of dihomono- γ -linolenic and timnodonic acid. In addition, timnodonic acid is a poor substrate for the cyclooxygenase system⁷⁵ (see *below*). As a consequence, prostaglandins from the 2-series are most abundantly produced.

Once released from the endogenous phospholipid pool by the action of phospholipase A₂, arachidonic acid may immediately be oxidized by various enzyme systems, such as *cyclooxygenase*, which converts this fatty acid to the endoperoxide PGG₂ (Fig. 1.3). This endoperoxide is subsequently reduced to PGH₂. PGH₂, in turn, serves as the substrate for various prostaglandin synthases, which convert it to the corresponding prostaglandins. In connective tissue mast cells, PGH₂ is predominantly converted into prostaglandin D₂ (PGD₂), through the action of PGD synthase.⁴⁵ Other cyclooxygenase products derived from arachidonic acid, such as thromboxane B₂, 6-keto-PGF_{1 α} and PGF_{2 α} , are also released by mast cells, but only in minor amounts.⁴⁵ Besides by the cyclooxygenase pathway, arachidonic acid can also be oxygenated at different sites by specific *lipoxygenases*, resulting in the formation of hydroxy fatty acids (HETEs). Special attention needs to be paid to 5-lipoxygenase, which is responsible for the conversion of arachidonic acid into 5-HPETE (5-hydroperoxyeicosatetraenoic acid). This compound may be converted into 5-HETE (5-hydroxyeicosatetraenoic acid) or into leukotriene A₄ (LTA₄). In mucosal mast cells, the predominant arachidonic acid-derived compound released is LTC₄. This is in contrast to connective tissue mast cells, in which PGD₂ is the predominant prostanoid released (also see Table 1.1.). The bronchoconstrictive actions of LTC₄, together with those of histamine are known to be responsible for many of the clinical symptoms of asthma. By the action of various peptidases, LTC₄ may be converted into other leukotrienes, such as LTD₄ and LTE₄, compounds which are less biologically active than the parent leukotriene, LTC₄. The (patho-)physiological effects of arachidonic acid-derived metabolites are numerous and are briefly summarized in Table 1.3.

Figure 1.3: Biosynthesis of prostanoids, hydroxy fatty acids and leukotrienes

Table 1.3 Selected (Patho-)physiological Effects of Several Arachidonic Acid Metabolites

Cyclooxygenase products	Action
HHT	Chemotaxis
PGI ₂	Inhibition of platelet aggregation
	Bronchodilation
PGF ₂ α	Bronchoconstriction
	Increased mucus secretion
PGE ₂	Increased vascular permeability
	Vasodilation
Lipoxygenase products	
HHT	Chemotaxis
5-HETE	Chemotaxis
LTC ₄	Smooth muscle constriction
	Increased vascular permeability
	Chemotaxis

Adapted from Metcalfe et al.⁶⁹

1.4 Biochemical Events Involved in Mast Cell Activation

Mast cells in the peritoneal cavity of rats can easily be obtained by lavage. In contrast, the isolation of mast cells from tissues such as the skin and heart, requires enzymatic digestions and usually results in low yield and high impurity. In addition, functional responses of isolated tissue mast cells are often severely impaired. Since peritoneal mast cells share many similarities with those found in tissues, these cells are widely used instead.⁹⁴ Because of their large size and since the degranulation process can easily be followed under the microscope, peritoneal mast cells have widely been used in patch-clamp studies. Peritoneal mast cells may be activated by activated by two different pathways: the antigenic pathway, involving cross-linking of IgE-antibodies, and the non-antigenic pathway. The latter pathway is induced by naturally occurring peptides such as complement factor C3a, or by synthetic peptides such as compound 48/80 and substance P.

1.4.1 The Antigenic Pathway

Immunological activation of mast cells occurs through cross-linking of IgE, bound to high-affinity $F_{c\epsilon}RI$ receptors ($F_{c\epsilon}RI$) by the F_c chain. The $F_{c\epsilon}RI$ contain a glycosylated α -chain exposed on the cell surface, a single β -chain, and two disulphide-linked γ -chains. The interaction between IgE and its receptor is highly specific and does not result in mast cell activation: it is the cross-linkage of two IgE antibodies by antigen or by other compounds such as lectins, which initiates a series of biochemical events, ultimately resulting in activation of the exocytotic and eicosanoid synthesizing process.⁴³ Antigen cross-linking results in the hydrolysis of phosphatidylinositol biphosphate by phospholipase C (PLC), leading to the generation of diacylglycerol (DAG) and inositol (1,4,5) trisphosphate ($Ins(1,4,5)P_3$). DAG activates protein kinase C (PKC) which, in turn, phosphorylates various cellular proteins. $Ins(1,4,5)P_3$ binds to a specific receptor on the endoplasmic reticulum, which results in mobilization of internal Ca^{2+} . PLC may be activated by at least two different pathways, one involving G proteins and the other involving receptor or non-receptor protein tyrosine kinases. PLC activated by G proteins is commonly referred to as PLC_β , whereas PLC activated by tyrosine kinases is commonly referred to as PLC_γ .^{57,85} The sequence of intracellular signal transduction events ultimately results in phosphorylation of cytoskeletal proteins involved in the translocation of secretory granules to the plasma membrane and activation of phospholipase A_2 . The individual granule membranes then fuse with each other and with the plasma membrane, which results in exposure of the granule matrix to the extracellular environment. Activation of PLA_2 results in the release of fatty acids, such as linoleic acid and arachidonic acid, from membrane phospholipids, which may be used as substrates for cyclooxygenase and/or lipoxygenase. Once formed intracellularly, prostanoids and hydroxy fatty acids are released into the extracellular environment by an as yet unknown mechanism.

1.4.2 The Non-antigenic Pathway

A number of cationic peptides and polyamines have the capacity to activate mast cells via the 'non-antigenic' pathway. This pathway of mast cell activation is restricted to connective tissue mast cells, such as rat peritoneal and human skin mast cells. Triggers include positively charged peptides, such as mastoparan⁷¹, neuropeptides such as substance P⁷⁰, peptidic hormones such as bradykinin¹⁷, natural and synthetic polyamines such as spermine and compound 48/80^{18,70}, respectively, and compounds belonging to the blood complement cascade, such as C3a.⁷³

Many attempts have been made to delineate the receptors or targets of these mast cell triggers. It has become clear that the non-antigenic pathway of mast cell activation is independent of selective receptors, but involves a direct interaction of these triggers with specific G proteins.⁵⁷ This interaction promotes a conformational change in specific G proteins, which results in activation of PLC β . Similar to the antigenic pathway, this is accompanied by the mobilization of Ca²⁺ from internal stores and activation of protein kinase C, respectively. These intracellular changes, together with an increase of the Ca²⁺ concentration, ultimately result in initiation of the degranulation process and activation of PLA₂. It should be noted that the activation of G proteins by peptides is mechanistically similar to their activation by receptors.^{40,72} Therefore, peptides and polyamines are generally used in studies on receptor/G protein interactions.

1.5 Mast Cells and the Cardiovascular System

1.5.1 Localization and (Patho-)physiological Roles

The heart contains a resident population of mast cells, located in the interstitium in the close vicinity of endothelial cells of small coronary vessels.^{79,90} Circulating histamine is taken up by the heart and is predominantly stored in mast cells.⁶³ Therefore, the release of this compound is generally used as a marker to indicate the extent of mast cell degranulation.

Over the past three decades, it has become clear that in the heart, similar to most other organs, the restoration of aerobic perfusion after a period of ischemia, may induce additional injury.³⁸ Besides an accelerated tissue necrosis, this, so-called 'reperfusion-induced injury', may also lead to a variety of other unfavourable events such as arrhythmias, ventricular fibrillations and stunning. The mechanisms underlying these phenomena have only partly been elucidated. Some possible causes are depletion of high-energy phosphate stores³⁷, Ca²⁺ accumulation⁹², the activation of phospholipases⁴⁹ and the production of lysophospholipids.²¹ In recent years, much attention has been paid as to the role of toxic oxygen free radicals (e.g. superoxide anions and hydroxyl radicals) in reperfusion-induced injury. It is thought that free radicals, generated during early reperfusion, react with polyunsaturated fatty acids within membranes, which results in loss of cellular function and integrity.²²

In the isolated rat heart, reactive oxygen metabolites have been shown to induce massive mast cell degranulation.¹⁰⁰ These findings are in agreement with early

observations of Anrep *et al.* (1936)², who discovered the release of histamine from the heart after hypoxemia in a canine heart-lung preparation. Since then, release of cardiac histamine has been found after coronary artery occlusion in dogs^{59,68}, global ischemia in the isolated guinea pig heart^{66,67} and after complement 5a-induced myocardial ischemia in domestic pigs.⁴⁷ Since mast cell degranulation may result in the release of potent harmful chemical mediators, such as proteinases, peroxidases and lysosomal enzymes, these observations have raised the question as to which extent mast cell degranulation during reperfusion, might be involved in reperfusion-induced myocardial injury. Until now, the evidence for a role of mast cells in myocardial injury is mainly based on the protective effects of the mast cell stabilizer lodoxamide tromethamine (U-42585E). For instance, Jolly *et al.*⁴⁸ have shown that in a canine model of myocardial injury, lodoxamide significantly reduced the extent of myocardial injury, measured as ultimate infarct size at 24 h postmortem. In addition, Keller *et al.*⁵⁰ have shown that in the isolated Langendorff-perfused rat heart, lodoxamide significantly reduced the release of both peroxidase (a proposed marker of mast cell degranulation) and creatine kinase (CK, a generally accepted marker of injury to cardiomyocytes) during reoxygenation.

Despite the widespread abundance of mast cells in the heart, surprisingly little is known about their possible (patho-)physiological roles. Since both histamine and heparin have been shown to enhance vascularization and endothelial cell proliferation in tissues such as the mesentery⁷⁶, changes in the number of cardiac mast cells in the interstitial space, and/or changes in their rate of secretory activity have been suggested to be involved in the regulation of the adaptive growth capacity of the coronary microcirculation during maturation and ageing of the heart.²⁷

1.5.2 Leukocyte-Endothelium Interactions: Role of Mast cells?

Extravasation of leukocytes is a multi-step process and a 'key-event' in inflammation. This process is initiated by the contact of leukocytes with the endothelium (*i.e.*, leukocyte rolling) and is followed by firm adhesion and extravasation (Fig. 1.4).^{19,60} Leukocyte rolling is characterized by rapid formation and subsequent breakage of bonds mediated by selectins.⁹ Selectins belong to a family of three proteins, named L (leukocyte)-, P (platelet)-, and E (endothelial)-selectin, according to the cell type on which they were firstly described. These proteins are known to mediate the adhesive interactions between leukocytes and the endothelium and between leukocytes and platelets in the blood.⁸⁸ L-selectin is constitutively expressed on most leukocytes, whereas E-selectin is synthesized and expressed by endothelial cells after cytokine activation.^{1,10} P-selectin is stored in α -granules of platelets and in Weibel-Palade bodies of endothelial cells, from where it can very rapidly be recruited to the plasma membrane by mediators such as histamine and thrombin.^{33,36} Each selectin recognizes specific carbohydrate sequences on either leukocytes (E-selectin or P-selectin) or the endothelium (L-selectin), and thereby mediates leukocyte-rolling. The transient nature of these adhesive interactions allows leukocytes to 'sample' the local endothelium for the presence of specific, additional, triggering factors that can activate leukocyte integrins (*see below*), which

allow the cascade to proceed. In the absence of such factors, the transient nature of selectin binding allows leukocytes to disengage and move on.

Figure 1.4: Sequential steps in adhesion of leukocytes to the endothelium

Leukocytes first 'roll' along the endothelium (1.), a process which is mediated by selectins. Activation of leukocyte integrins (2.) by PAF or various cytokines such as Interleukin-8, generated by underlying inflammatory cells (mast cells) or the endothelium itself, allows firm adhesion of leukocytes to the endothelium by interaction of leukocyte integrins with their corresponding counterparts on endothelial cells (3.). This interaction results in flattening of the leukocytes and is followed by extravasation of these cells under the influence of various chemotactic factors such as Interleukin-8 (4.).

Firm adhesion of leukocytes to the endothelium is mediated by a different set of adhesion molecules, called integrins. Integrins are a family of heterodimeric membrane glycoproteins that consist of an α and β subunit and can be grouped into subfamilies based on their β subunit. Most of the integrins require an activation step before they can bind to their respective ligands on the endothelium. Such an activation may occur through the release of platelet-activating factor (PAF) or by various interleukins such as interleukin-8 (IL-8), produced by the endothelium itself or by underlying inflammatory cells.¹⁰⁵ The most potent integrins for cell-cell interactions are the $\beta 1$ integrins, such as VLA-4 (Very Late Antigen-4), and the $\beta 2$ integrins (leukocyte cell-adhesion molecules), such as Mac-1 and LFA-1. VLA-4 is present on cells such as lymphocytes and monocytes and mediates binding of these cells to the endothelial adhesion receptor Vascular Cell Adhesion Molecule-1 (VCAM-1).⁸⁹ LFA-1 is present on the surface of virtually all circulating leukocytes, whilst Mac-1 has a more limited distribution, being present on neutrophils, monocytes, macrophages, eosinophils and large granular lymphocytes.⁵⁴ $\beta 2$ Integrins such as Mac-1 and LFA-1, bind to the InterCellular Adhesion Molecule 1 (ICAM-1). Expression of VCAM-1, like that of ICAM-1, on vascular endothelium is induced by proinflammatory cytokines such as $\text{TNF}\alpha$.⁸ Contacts like Mac-1:ICAM-1 or VLA-4:VCAM-1 result in flattening of the leukocytes and provide the basis for firm adhesion of these cells to the endothelium. Thereafter, the flattened leukocytes

transmigrate into the extracellular matrix tissue by moving between endothelial cell junctions under the influence of chemotactic factors such as IL-8.

Mast cells may release a number of inflammatory mediators such as histamine, $\text{TNF}\alpha$, platelet activating factor (PAF) and various interleukins, such as IL-8.³¹ Therefore, it has been speculated that tissue mast cells may contribute to events, ultimately resulting in leukocyte-adhesion and transmigration. In mice, degranulation of cutaneous mast cells by IgE or compound 48/80 has been shown to cause neutrophil infiltration, a phenomenon which was markedly reduced in mast cell-deficient mice.^{95,103} In addition, it has been shown that superfusion of the hamster cheek pouch with compound 48/80 caused leukocytes to leave the vasculature and migrate toward degranulating mast cells.⁸² In the heart *in vivo*, the infiltration of leukocytes in flow-deprived areas is known to increase ischemia/reperfusion-induced myocardial injury.^{26,61,87} Leukocytes may contribute to this kind of injury by the release of harmful substances such as proteases, oxygen free radicals and hydrogen peroxide by leukocytes, which may directly injure cardiac muscle cells.⁶² Until now, information about the role of mast cells in the extravasation of leukocytes in the heart is lacking.

1.6 Aims and Outline of the Thesis

In the present thesis, various aspects of mast cell activation and mediator release will be addressed. Special attention will be paid to the role of mast cells in the cardiovascular system. Since the isolation of mast cells from cardiac tissue is laborious and usually results in low yield and purity, we developed a method to isolate mast cells in high quantity from the peritoneal cavity. Cardiac mast cells, similar to peritoneal mast cells, belong to the connective tissue type. In order to quantitate the extent of mast cell degranulation, a fast and reliable high-performance liquid chromatographic (HPLC) method for the determination of histamine in biological samples was developed. Most of the HPLC techniques for the determination of histamine described until now make use of o-phthalaldehyde (OPA) as the derivatizing agent. Since OPA-derivatives are relatively unstable and require strict conditions for derivatization, we developed a method using fluorescamine as derivatizing agent, which resulted in relatively stable histamine-derivatives. The technique to isolate mast cells from the peritoneal cavity, as well as the HPLC technique and several of its applications are presented in **Chapter 2**.

When incorporated in phospholipids, certain long-chain, highly unsaturated fatty acids are not only important constituents of cellular membranes, but may also serve as substrates for the synthesis of prostanoids and hydroxy fatty acids. We were interested to know to which extent in mast cells the release of prostanoids and hydroxy fatty acids was influenced by diet-induced changes in membrane fatty acid composition. Therefore, peritoneal mast cells were isolated from rats which had been fed diets that differed in their fatty acid composition. The results of this study are presented in **Chapter 3**.

Studies in rat platelets have indicated that the type of dietary fat might also influence specific cellular processes and functions.^{39,44} We studied to which extent in mast cells, the extent of mast cell degranulation was influenced by the fatty acid composition of membrane phospholipids. Therefore, mast cells, isolated from diet-

modified rats were activated with various concentrations of the G protein-activating compound 48/80. The results of this study are presented in **Chapter 4**.

A large number of studies have provided a great deal of information about specific steps of the signal transduction cascade, ultimately resulting in exocytosis and activation of the eicosanoid synthesizing pathway. However, the exact role of cytosolic calcium and protein tyrosine kinases in the release of histamine and PGD₂ by these cells is not yet known. This was studied in peritoneal mast cells using a specific fluorescent Ca²⁺ probe and inhibitors of protein tyrosine kinases. The results of this study are presented in **Chapter 5**.

Indirect evidence from *in vivo* and *ex vivo* studies has indicated that mast cells are involved in acute injury to cardiomyocytes evoked by ischemia/reperfusion or hypoxia/reoxygenation. We investigated the role of mast cells in acute ischemia/reperfusion-induced myocardial injury in a direct way. Therefore, Langendorff-perfused hearts, isolated from sensitized and non-sensitized rats, were subjected to ischemia/reperfusion. During normoxic perfusion, preceding ischemia/reperfusion, hearts were administered a bolus-injection of antigen, known to result in a massive mast cell degranulation in sensitized hearts.¹⁰¹ Both control and 'mast cell depleted hearts' were subjected to ischemia/reperfusion. Lactate dehydrogenase (LDH) was used as a marker to indicate the extent of (irreversible) injury to cardiomyocytes. The results of this study are presented in **Chapter 6**.

In **Chapter 7**, the role of mast cells in another model of myocardial injury is described. Isolated, Langendorff-perfused hearts were subjected to hypoxia/reoxygenation. Histamine was used as a marker for mast cell degranulation, whereas LDH and creatine kinase (CK) were used to indicate the extent of injury to cardiomyocytes. Special attention was paid to the use of peroxidase (PO) as a potential marker of mast cell degranulation.

Various studies in tissues other than the heart have indicated that products released from mast cells are involved in the process of leukocyte rolling/adhesion and/or extravasation.^{53,55,82,103} We approached the role cardiac mast cells in the expression of endothelial leukocyte-adhesion molecules in the intact heart with an *in vitro* rat experimental model. Peritoneal mast cells were studied for their capacity to induce the expression of leukocyte-adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin, by cultured endothelial cells. The results of this study are described in **Chapter 8**.

Finally, the data obtained in various studies described in this thesis are summarized and placed in a broader perspective in **Chapter 9**.

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CHAPTER 2

A High-Performance Liquid Chromatographic Method for the Determination of Histamine in Biological Samples:

An improved Technique to Isolate Peritoneal Mast Cells

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*Charles M.C.J. van Haaster, Wim Engels, Paul J.M.R. Lemmens, Gerard Hornstra and
Ger J. Van der Vusse*

Rapid and highly sensitive high-performance liquid chromatographic method for the determination of histamine and 3-methyl histamine in biological samples using fluorescamine as the derivatizing agent

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2.1 ABSTRACT

A highly sensitive and rapid HPLC assay for the determination of histamine and 3-methyl histamine in biological samples using 1-methyl histamine as the internal standard is described. Samples were purified and concentrated on cation-exchange columns and derivatized with fluorecamine. The lower detection limit was 20 pg on column. Linearity was demonstrated up to 20 ng on column. The samples could be derivatized simultaneously before injection on HPLC and were stable for at least 7 days. The method was used for the determination of histamine and related compounds in coronary effluents, extracts of homogenized rat hearts and supernatants from stimulated peritoneal mast cells. In addition, a simple method to isolate mast cells in high purity from the peritoneal cavity is also described.

2.2 INTRODUCTION

Histamine, stored in secretory granules of mast cells, is known to be responsible for some of the clinical symptoms of immediate hypersensitivity reactions and asthma.¹⁶ Upon activation, histamine is rapidly released into the extracellular environment. Therefore, this compound is generally used as a marker to indicate the extent of mast cell degranulation. A number of analytical methods for the determination of histamine have been described. These include bioassays¹, fluorometry^{9,21}, radioenzymatic assays^{4,5} and gas chromatography-mass spectrometry.^{11,15} Most of these techniques, however, suffer from disadvantages such as low specificity and sensitivity, and the possible appearance of contamination. Therefore, various methods to determine histamine by High Performance Liquid Chromatography (HPLC) have been developed, which give accurate and rapid results.^{2,3,17-19} Most of these methods make use of o-phthalaldehyde (OPA) as the derivatizing agent, which results in histamine-derivatives which are rather instable. This has the disadvantage that rather sophisticated and expensive HPLC equipments, for instance those using on- or post-column derivatization, are required. We developed a method for the determination of histamine in biological samples, which resulted in relatively stable derivatives and which required only an ordinary HPLC apparatus. Since concentrations of histamine in biological samples, for instance in coronary effluents (*see Chapter 7*), may be very low, a concentration-step prior to analysis was introduced.

In vivo, trauma and lethal stress in rats may result in the formation of 3-methyl histamine from histamine.⁸ In order to quantitate the possible formation of 3-methyl histamine in rat heart, this compound was also included in the present method. The method was used to determine the content of histamine and 3-methyl histamine in rat heart homogenates and coronary effluents. In addition, the technique was used to determine the histamine content of peritoneal mast cells.

In most of the techniques for the isolation of peritoneal mast cells described until now, erythrocytes usually contaminate the final mast cell preparation, which might influence the outcome of studies with preparations assumed to be pure. Since the presence of erythrocytes in the mast cell preparation might result in erroneous data

concerning the fatty acid composition of mast cell phospholipids (see Chapter 3), a method to separate these cells from mast cells was developed. This technique is also described in this chapter.

2.3 MATERIALS AND METHODS

Chemicals

Histamine, 3-methyl histamine and 1-methyl histamine were obtained from Sigma (St. Louis, USA). Bakerbond Carboxylic acid (COOH) 40 μm , 60 Å, bulk package, was obtained from Baker Inc., (Philipsburg, USA). $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, HCl (Suprapur®), gelatin, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, Na_2EDTA , NH_4OH , $\text{o-H}_3\text{PO}_4$ and HClO_4 (all analytical grade) were obtained from Merck (Darmstadt, FRG). Acetonitrile and methanol (HPLC-grade) were purchased from Rathburn (Walkerburn, UK). Triethylamine, purified before use on short tip-plugged pasteur pipettes filled with Al_2O_3 (ICN Biomedicals Inc., Costa Mesa, USA), was obtained from Janssen Chimica (Tilburg, the Netherlands). Fluorescamine was from Fluka AG (Buchs, Switzerland). Metrizamide® was purchased from Nyegaard & Co. (Oslo, Norway). Heparin (Thromboliquine®) was obtained from Organon Teknika (Boxtel, the Netherlands). Water was purified with a milli-Q purification unit from Millipore (Bedford, USA). Compound 48/80 (a condensation product of N-methyl-p-methoxy-phenethylamine with formaldehyde) was a kind gift from dr. M. Borgers (Janssen Pharmaceutica, Beerse, Belgium).

Purification and Concentration of the Samples

Small columns were prepared with a slurry of Bakerbond Carboxylic acid material in methanol (150 mg/column) in pasteur pipettes tip-plugged with glassfibre prefilter material (Satorius, Göttingen, FRG). The columns were washed with 2 ml of H_2O followed by 2 ml of 1M HCl, 2 ml of H_2O and 4 ml of 0.2 M sodium phosphate buffer, pH 6.4. After addition of 500 ng of 1-methyl histamine as internal standard, samples (brought to pH 6.4 with phosphate buffer) were applied onto the columns. Columns were washed with 4 ml of 50 mM Na_2EDTA , pH 6.4 and 4 ml of H_2O . Histamine, 3-methyl histamine and 1-methyl histamine were eluted with 1 ml of 1M HCl. The eluate was dried under a stream of nitrogen at 40 °C for approximately 15 min and the residue was subsequently dissolved in 100 μl of H_2O .

Derivatization

To the purified samples, 400 μl of 50 mM sodium borate-buffer, pH 9.1 was added. Under continuous, vigorous stirring, 500 μl of a freshly prepared fluorescamine solution in acetonitrile (20 mg/100 ml) was added. After stirring for 1 min, the derivatized samples were dried under a stream of nitrogen at 40 °C for approximately 15 min. The residues were dissolved in 1 ml of the HPLC mobile phase (see below).

Fluorescamine reacts with compounds possessing a primary amino group almost instantaneously at room temperature in aqueous solution at pH 7.5 to 9.¹² The excess of fluorescamine is rapidly hydrolyzed ($t_{1/2}$ is about 10 seconds) to yield a water-soluble,

non-fluorescent, product. The derivatization reactions of histamine, 1-methyl and 3-methyl histamine with fluorescamine are shown in Fig. 2.1.

Figure 2.1: Derivatization of histamine, 1-methyl or 3-methyl histamine with fluorescamine®

HPLC Instrumentation

The HPLC system consisted of a Spectroflow 400 pump (Kratos Analytical, Ramsey, USA), a Promis auto-injector (Spark Holland, the Netherlands) with a Rheodyne 7010 injection valve and a Perkin-Elmer LS-1 fluorescence detector (Beaconsfield, UK) with a reversed-phase C18-column, 250 x 4 mm I.D., 5 μ m particles (Chrompack, Middelburg, the Netherlands). The injection volume was 20 μ l and the flow rate 0.7 ml/min. The fluorescence detector had the following settings: excitation wavelength, 360 nm; emission wavelength, 440 nm. The temperature of the HPLC column was kept at 20 °C. Samples were kept at 4 °C in the auto-injector device in the dark. Data were acquired with Rainin software, using the Dynamax program for integration of the chromatograms (Rainin Instrument Co., Ridgefield, USA).

Chromatographic Conditions

The HPLC mobile phase consisted of H₂O/acetonitrile/triethylamine/o-H₃PO₄ (850/150/4/4, v/v) brought to pH 6.4 with NH₄OH. In order to obtain an optimal response of the fluorescence detector and a low noise level, the mobile phase was gassed daily with helium (5 min at a flow rate of 250 ml/min for each 1000 ml of the mobile phase).

Assessment of Linearity, Stability, Reproducibility and Recovery

The relationship between the amounts of fluorescamine-derivatized histamine or 3-methyl histamine injected on column and their relative peak areas was assessed as follows. To 1 ml 0.2 M phosphate buffer (pH 6.4), various amounts of histamine and 3-methyl histamine standard solutions (0, 100, 200, 300, 400, 500, 600 and 700 ng, each amount prepared in triplicate) were added. To all samples 500 ng 1-methyl histamine was added. After concentration and derivatization of the samples, the derivatives were dissolved in 1 ml of the HPLC mobile phase. Following injection of 20 μ l of the samples (corresponding to 0 to 14 ng on column) and separation by HPLC, absolute peak areas of histamine and 3-methyl histamine were transformed to relative peak areas by correction for the internal standard. Samples were immediately analyzed after derivatization (day 0) and re-analyzed on day 1 and 7.

For the determination of the stability, samples ($n=12$) from a standard solution, containing histamine (300 ng), 3-methyl histamine (300 ng) and 1-methyl histamine (250 ng), were concentrated, derivatized and analyzed by HPLC on day 0 and day 7.

In order to determine the reproducibility, two standard solutions (I and II) were prepared. Standard solution I contained 1500 ng/ml histamine, 1500 ng/ml 3-methyl histamine and 3000 ng/ml of the internal standard. Standard solution II contained 6000 ng/ml histamine, 6000 ng/ml 3-methyl histamine and 3000 ng/ml of the internal standard. On three consecutive days, 100 μ l samples ($n=6$) were withdrawn from solutions I and II. Samples were brought onto the cation-exchange columns in 2 ml 0.2 M sodium phosphate buffer (pH 6.4). After sequential wash-steps and elution with HCl, samples were dried, redissolved in borate buffer, derivatized with fluorescamine and immediately analyzed by HPLC.

To determine the recovery of the purification/concentration procedure, 100 μ l samples from solution I ($n=10$) were immediately derivatized with fluorescamine, solubilized in 1 ml of the mobile phase and analyzed by HPLC. Absolute peak areas of these samples were compared to the absolute peak areas from the samples concentrated on cation-exchange columns.

Samples Rat Heart Homogenates

Histamine and 3-methyl histamine were determined in heart homogenates from Lewis rats ($n=9$). In order to remove blood from the tissue, hearts were perfused for 5 min according to Langendorff.¹³ After heparinization (1000 IU/kg, intraperitoneally), hearts were isolated and perfused according to Langendorff at a constant pressure of 80 mm Hg. The perfusion fluid was Krebs-Ringer's solution (pH 7.4) containing (in mM): NaCl (130), KCl (5.6), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), $NaHCO_3$ (28.6), $CaCl_2$ (2.2), and glucose (11.1). The buffer was kept at 37 °C and was equilibrated with 95% O_2 /5% CO_2 (v/v) throughout the experiment, which always resulted in a final pO_2 >80 kPa. After 5 min perfusion was stopped and parts (about 200 mg) of the ventricular tissue were brought into ice-cold 50 mM Tris.HCl buffer (pH 8.5) to a final concentration of 5% (w/v). After addition of the internal standard, hearts were minced and subsequently homogenized with the use of an Ultra-Turrax (Wilten Woltil, De Bilt, the Netherlands). Thereafter, samples were sonicated (30 Watt, amplitude 12 μ , peak to peak) with an MSE sonifier

(Crawley, UK) for 2 min with repeated intervals of 5 s. During homogenization and sonication, samples were kept on ice. After addition of HClO_4 to a final concentration of 0.3 M, samples were boiled for 5 min at 100 °C. Thereafter, samples were neutralized with KOH, cooled to 0 °C and centrifuged at $2,000 \times g$ for 5 min (4 °C) to remove the precipitate of KClO_4 . Supernatants were collected and stored at -20 °C until analysis.

Samples of Coronary Effluents

In a subset of experiments, histamine and 3-methyl histamine were determined in coronary effluents collected from hearts from Lewis rats ($n=3$). After 5 min of normoxic perfusion, hearts were administered a bolus-injection of 50 μg of compound 48/80 (dissolved in 0.5 ml perfusion buffer) in order to induce mast cell degranulation. Coronary effluents were collected during the following period of 1 min.

Purification of Peritoneal Mast Cells

Peritoneal mast cells were isolated according to a modified version of the procedure of Schwartz *et al.*²⁰ Rats were anaesthetized with diethyl ether and 20 ml of lavage buffer, composed of 136 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , gelatin (0.1%, w/v) and heparin (10 I.E./ml), pH 7.4, was injected intraperitoneally. The abdomen was massaged for about 1 min and the peritoneal exudate was recovered. After centrifugation at $195 \times g$ for 5 min, cells were washed twice with lavage buffer and resuspended into a final volume of 1 ml. The cell suspension was layered onto 2.5 ml of a 22.5% metrizamide gradient (w/v) in lavage buffer (Fig. 2.2) and centrifuged at $470 \times g$ for 15 min. This resulted in a complete separation of mast cells and erythrocytes from other nucleated cells. After carefully removing the interphase, the sediment was washed twice with lavage buffer and resuspended into a final volume of 1 ml. Since erythrocytes have a density range ($d=1.10$ to 1.12) which overlaps the lower range of densities of mast cells ($d=1.09$ to 1.17), another step is needed to completely separate these cells. Therefore, a velocity gradient was introduced, which allowed separation of the cells by their differences in cell size rather than in density.⁶ This was achieved by layering the cell suspension (1 ml) onto a two-step 3%/9% (w/v) metrizamide gradient. This gradient was centrifuged at $50 \times g$ for 10 min. This resulted in an interphase containing erythrocytes, and a sediment containing mast cells. The sediment was washed twice with buffer pH 7.4, containing 136 mM NaCl, 2.7 mM KCl, 10 mM Hepes, 2 mM MgCl_2 , 5 mM glucose and 0.1% (w/v) bovine serum albumin (fatty acid free), and were finally resuspended in this buffer to a concentration of $5 \cdot 10^5$ cells/ml. Mean mast cell count was about $8 \cdot 10^5$ cells per rat. Cytocentrifuge preparations stained with 0.1% toluidine blue (w/v) revealed a highly homogenous mast cell population with a purity higher than 99%. Additional staining with May-Grünwald-Giemsa showed that platelets and erythrocytes were absent and that only a single mononucleated cell was present on a mast cell count of 10^5 cells. Mast cells were counted using a Coulter counter (model ZM, Coulter Electronics GmbH, Krefeld, FRG).

Figure 2.2: Two-step purification of peritoneal mast cells from a peritoneal lavage

Activation of Peritoneal Mast Cells

Aliquots (100 μ l) of the mast cell suspension (10^6 cells/ml) were brought into 900 μ l of buffer pH 7.4. After preincubation at 37 $^{\circ}$ C for 5 min, cells were stimulated with compound 48/80 (10 μ g/ml) in the presence of 1 mM CaCl_2 ($n=5$). After 5 min, 500 μ l samples were withdrawn from the incubations, immediately layered onto 250 μ l of silicon oil ($d=1.049$) and centrifuged in an Eppendorf centrifuge for 20 s at 15,000 $\times$ g. The supernatants were collected for the determination of histamine. Supernatants from unstimulated cells were also analyzed. Cells were incubated with 0.1% Triton X-100 (v/v) to determine the total mast cell histamine content. After addition of 500 ng of the internal standard, samples were stored at -20 $^{\circ}$ C until analysis.

2.4 RESULTS

Linearity, Stability, Reproducibility and Recovery

The linearity of the fluorescence intensity versus the amounts of histamine and 3-methyl histamine was evaluated by analyzing a range from 0 to 700 ng histamine and 3-methyl histamine per sample (corresponding to 0 to 14 ng on column). Plots of relative peak areas of histamine and 3-methyl histamine versus the amounts derivatized were linear (Fig. 2.3). Regression equations of relative peak areas (Y) versus the amounts (on column) of derivatized histamine and 3-methyl histamine (X) were: $Y = 0.090 X + 0.056$ ($r = 0.996$) and $Y = 0.116 X + 0.031$ ($r=0.999$), respectively. Since the samples were individually concentrated, derivatized and analyzed, this implies that the procedure has a constant efficiency which is independent of the amount of histamine and 3-methyl histamine derivatized over the range selected. In practice, linearity was demonstrated up to 20 ng on column for both histamine and 3-methyl histamine (*data not shown*). Assuming a signal to noise ratio of 3:1, lower detection-limits of histamine and 3-methyl histamine were about 20 pg on column. Analysis of a standard solution containing 300 ng histamine and 3-methyl histamine in the presence of 250 ng of the internal standard (see Materials and Methods) on day 7 showed that only 10% of the fluorescence intensity was lost over a period of seven days. Absolute peak areas of histamine, 3-

methyl histamine and the internal standard on day 7 (in % of absolute peak areas on day 0) were $90.9 \pm 4.5\%$, $91.7 \pm 2.9\%$ and $90.9 \pm 2.6\%$, respectively.

Figure 2.3: Calibration curves for histamine and 3-methyl histamine

X: ng histamine (●) or 3-methyl histamine (▲) injected on column

Y: relative peak area (in arbitrary units, A.U.)

Derivatives of standard solutions used for the calibration curves were immediately analyzed upon derivatization and re-analyzed on day 1 and 7. Multiple analysis of variance revealed no significant differences between the relative peak areas of histamine and 3-methyl histamine on day 1, 2 and 7 ($\alpha=0.05$) for all the amounts used. The low coefficient of variation both for samples containing low or high amounts of histamine or 3-methyl histamine (Table 2.1) shows that these compounds can be detected with a high reproducibility. When the samples were concentrated on the cation-exchange columns, absolute peak areas were always >95% of those not concentrated on the columns, which implies that the recovery of the procedure was >95%.

Applications

The combination of purification of samples on cation-exchange columns followed by derivatization with fluorescamine was used to determine histamine and 3-methyl histamine in various biological samples. Fig. 2.4A represents a typical chromatogram of a standard solution containing 5 ng (on column) of histamine, 3-methyl histamine and the internal standard, showing that histamine elutes at 15 min, 3-methyl histamine at 20 min and the internal standard at 25 min. The three compounds were completely separated.

Table 2.1: Reproducibility Data for the Determination of Histamine and 3-Methyl Histamine in Standard Solutions

Concentration (ng/sample)	CV (%) ^{*)}			
	Within Day ^{**)}		Day to Day ^{***)}	
	Histamine	3-Methyl Histamine	Histamine	3-Methyl Histamine
150	3.2	3.3	2.9	2.7
600	6.7	6.9	6.7	5.9

Each sample (n=6 for each concentration) was concentrated on the cation-exchange columns, derivatized with fluorescamine and analyzed by HPLC on three days in a one week period. Standard solutions were prepared as described in Materials and Methods.

^{*)} The coefficient of variance (CV) was obtained by dividing the standard deviation by the mean
<sup>**) n=6
^{***) n=3}</sup>

Fig. 2.4B represents a typical chromatogram of a coronary effluent collected during a 1 min period after a bolus injection with 50 µg compound 48/80. As a consequence of mast cell degranulation, a net release of 1550 ± 150 ng (mean $\pm$ SD, n=3) of histamine in this time interval was observed. No 3-methyl histamine could be detected in the coronary effluent. In coronary effluents of non-stimulated hearts both histamine and 3-methyl histamine were undetectable. Fig. 2.4C shows a representative chromatogram of a rat heart homogenate. The presence of 3-methyl histamine in rat hearts could not be demonstrated. The mean amount of histamine in rat hearts was found to be 2.7 ± 0.3 µg/gram wet weight (mean $\pm$ SD, n=9). When rat heart homogenates (n=3) were spiked with 500 ng 3-methyl histamine, we observed mean amounts of 480 ± 20 ng, corresponding to 96% recovery of this compound. Stimulation of rat peritoneal mast cells with 10 µg/ml compound 48/80 (Fig. 2.4D) in the presence of 1 mM CaCl₂ resulted in the release of 19 ± 2 µg histamine/10⁶ cells (mean $\pm$ SD, n=5). Total histamine content was found to be 25 ± 3 µg/10⁶ cells (n=5), implicating that about 75% of total mast cell histamine was released with this agonist. Spontaneous histamine release, i.e., histamine release in the absence of agonist, was low ($4 \pm 1\%$). The amount of 3-methyl histamine in supernatants from stimulated peritoneal mast cells remained below the detection limit (i.e., <1 ng/10⁶ cells). Analysis of supernatants from stimulated peritoneal mast cells, rat heart homogenates and coronary effluents on day 1 and 7 resulted in identical values as those observed on day 0 (data not shown).

Figure 2.4: Typical reversed-phase chromatograms of standards and biological samples

(A) standard fluorecamine-derivatives of histamine (1), 3-methyl histamine (2) and the internal standard 1-methyl histamine (3), each peak representing 5 ng on column
 (B) coronary effluent from a heart stimulated with 50 μg of the mast cell degranulating agent compound 48/80
 (C) an extract of a rat heart homogenate
 (D) supernatant of rat peritoneal mast cells stimulated with 10 $\mu\text{g}/\text{ml}$ compound 48/80 in the presence of 1 mM CaCl_2

The Y-axis represents fluorescence (arbitrary units), the X-axis the retention time (min).

2.5 DISCUSSION

Most of the HPLC methods to determine histamine in biological samples described until now, make use of OPA as the derivatizing agent. Derivatization with this compound requires carefully controlled reaction conditions and duration of the derivatization reaction, and results in products which are relatively unstable ($t_{1/2}$ of about 3 hours, calculated from Saito *et al.*¹⁹). These properties require that the samples have to be analyzed immediately after derivatization or that derivatization takes place on- or post-column. The disadvantage of these procedures, where the mobile phase contains the OPA reagents, is that they require rather expensive additional equipments for the chromatographic system. In addition, the use of a post-column reagents results in significant peak broadening and dilution of the samples.¹⁹

In the present method, fluorescamine, frequently used in amino acid and peptide analysis, was used as the derivatizing agent. This compound, which in itself is not fluorescent, reacts only with primary amino groups of peptides almost instantaneously at room temperature in aqueous solution at pH 7.5 to 9.¹² The excess of fluorescamine is rapidly hydrolyzed ($t_{1/2}$ is about 10 seconds) to yield a water-soluble non-fluorescent product. It was found that absolute peak areas of histamine, 3-methyl histamine and 1-methyl histamine on day 7 were about 90% of those observed on day 0. This indicates that derivatization of samples with fluorescamine resulted in relatively stable derivative. Since the decrease in absolute fluorescence was equal for all three compounds tested, samples may be analyzed up to 7 days after derivatization without a significant loss of sensitivity. This notion was confirmed by the observation that, for all the amounts investigated, relative peak areas of histamine and 3-methyl histamine on day 1 and 7 were identical to those of day 0. Since the within-day as well as the day-to-day coefficients of variation were low, it can be concluded that the method has a good reproducibility. In addition, since absolute peak areas of samples, concentrated on cation-exchange columns, were always >95% of the samples not concentrated on the columns, the method allowed a concentration procedure without a substantial loss of material.

The present technique was used to assess the histamine and 3-methyl histamine content of various biological samples. It was found that administration of compound 48/80 to normoxic perfused isolated rat hearts resulted in a massive mast cell degranulation, as indicated by the release of about 60% of cardiac histamine in the first min after administration. Similar values have been found upon anaphylactic degranulation of sensitized rat hearts.²² Total histamine in rat hearts was found to be 2.7 ± 0.4 $\mu\text{g/g}$ wet weight, which is in agreement with results from Kasziba *et al.*¹⁰ Addition of compound 48/80 (10 $\mu\text{g/ml}$) and CaCl_2 to rat peritoneal mast cells resulted in the release of 75% of total mast cell histamine, a value which has also been described earlier by others.^{7,14,23} The observation that the amounts of 3-methyl histamine in coronary effluents and cardiac tissue were below detection limits, show that under normal conditions, this metabolite is not present in cardiac tissue and that it is not released/formed after compound 48/80-induced mast cell degranulation. Similarly, it can be concluded that 3-methyl histamine is not constitutively present in

peritoneal mast cells and is not released/formed upon compound 48/80-induced degranulation.

In conclusion, the described method allows the determination of histamine and 3-methyl histamine in biological samples using a simple and highly sensitive isocratic HPLC method. It was found that derivatization with fluorescamine resulted in samples which were stable for at least 7 days. The present method has the advantage that all samples can be derivatized simultaneously and that analysis can be carried out in a simple, automated low-cost HPLC system. We propose that the present HPLC method provides a useful tool to determine histamine and 3-methyl histamine in biological samples.

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CHAPTER 3

Formation of Prostanoids and Hydroxy Fatty Acids by Stimulated Rat Peritoneal Mast Cells: Role of the Dietary Fat Type

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Formation of Prostanoids and Hydroxy Fatty Acids by Stimulated Rat Peritoneal Mast Cells: Role of the Dietary Fat Type

3.1 ABSTRACT

In order to gain insight into the role of mast cell membrane fatty acid composition on the formation of prostanoids and hydroxy fatty acids, peritoneal mast cells were isolated from rats which were fed diets differing in their fatty acid composition: (i) mackerel oil (MO), abundant in (n-3) fatty acids, (ii) sunflowerseed oil (SO), rich in (n-6) fatty acids, particularly linoleic acid, and (iii) hydrogenated coconut oil (HCO), rich in saturated fatty acids. The introduction of (n-3) fatty acids in the diet resulted in the incorporation of timnodonic acid, 20:5(n-3), clupanodonic acid, 22:5(n-3), and cervonic acid, 22:6(n-3), in mast cell phospholipids. This was accompanied by a significant decrease in the contents of arachidonic acid, 20:4(n-6), and one of its elongation products, adrenic acid, 22:4(n-6). The arachidonic acid content was highest in mast cell phospholipids from the HCO-group. The high content of linoleic acid in the SO-diet resulted in an increased incorporation of this fatty acid in mast cell phospholipids. Upon activation of the cells with the calcium ionophore A23187, major products released were PGD₂, PGE₂, HHT, 9-HODE and 13-HODE. Minor products released from these cells were 6-keto-PGF_{1α}, PGF_{2α}, TxB₂, 15-HETE and 11-HETE. In general, the formation of prostanoids and hydroxy fatty acids increased with the precursor fatty acid content in mast cell membranes. For instance, the low arachidonic acid content in mast cell phospholipids from the MO-group was accompanied by a relatively low formation of PGD₂ and PGE₂. The high linoleic acid content in mast cell phospholipids from the SO-group was accompanied by a high release of 9-HODE and 13-HODE, cyclooxygenase and lipoxygenase products from linoleic acid, respectively. The results suggest that in mast cells, dietary fatty acids are able to modulate the formation of prostanoids and hydroxy fatty acids by changing the availability of precursor fatty acids. We propose that diet-induced changes in the production of prostanoids and hydroxy fatty acids may have important consequences in biological processes in which these mast cell-derived products are involved.

3.2 INTRODUCTION

Eicosanoids (cyclooxygenase and lipoxygenase products containing 20-carbon fatty acids) are important mediators of inflammation and allergy.^{1,7} In mast cells, activation of phospholipase A₂ (PLA₂) results in the release of arachidonic acid, 20:4(n-6), the major substrate for eicosanoid synthesis, from membrane phospholipids. Arachidonic acid may subsequently be metabolized by cyclooxygenase to form prostaglandins and thromboxanes (also called: prostanoids), and by lipoxygenase to form hydroxy fatty acids (HETEs). In addition, arachidonic acid may be converted by cyclooxygenase to form 12-hydroxyheptadecatrienoic acid (HHT). Activation of PLA₂ also results in the liberation of linoleic acid, 18:2(n-6), which, in turn, may be metabolized by cyclooxygenase and lipoxygenase to form 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE), respectively.

In various cell types, such as platelets, changes in the type of dietary fat have been shown to affect the fatty acid composition of membrane phospholipids.¹³ Since some

of these fatty acids serve as precursor fatty acids for the synthesis of prostanoids and hydroxy fatty acids, diet-induced changes in phospholipid fatty acid composition play an important role in the amount and/or type of prostanoid or hydroxy fatty acid produced. Until now, it is unknown to which extent the formation of prostanoids and hydroxy fatty acids is related to the fatty acid composition of mast cell phospholipids. In order to study this, peritoneal mast cells were isolated from rats which had been fed diets differing in their fatty acid composition.

It was found that, in general, an increased availability of arachidonic acid or linoleic acid in mast cell phospholipids was accompanied by an increased formation of prostanoids and hydroxy fatty acids, such as PGD₂ and 9-HODE, respectively. The results suggest that in mast cells, dietary fatty acids are able to modulate the formation of prostanoids and hydroxy fatty acids by changing the availability of the precursor fatty acids in membrane phospholipids.

3.3 MATERIALS AND METHODS

Animals

Wistar rats of 3 weeks old, weighing about 120 g, were obtained from a specified pathogen-free colony of the Charles River Laboratory (Sulzfeld, FRG). All animals were kept in stainless steel cages (1 rat per cage) and had free access to food and water. Experiments were performed according to institutional guidelines.

Diets

Animals were randomly divided into 3 groups and were fed, *ad libitum*, adequate diets containing 50 energy% (en%) fat (Table 3.1), but which differed in their fatty acid composition:

1. *MO-diet*: 45 en% mackerel oil (GSO) (supplier: Norwegian Herring Oil and Meal Industries, Fijllingdalen, Norway), rich in long-chain (n-3) polyunsaturated fatty acids, 20:5(n-3) and 22:6(n-3) in particular, supplemented with 5 en% sunflowerseed oil (SO) to ensure an adequate supply of (n-6) essential fatty acids
2. *SO-diet*: 50 en% sunflowerseed oil, rich in (n-6) fatty acids, 18:2(n-6) in particular
3. *HCO-diet*: 45 en% hydrogenated coconut oil (HCO), rich in saturated fatty acids, supplemented with 5 en% SO.

The SO and HCO-diets were freshly prepared once a week, the MO-diet at least 3 times a week. All diets were stored at 4 °C. The fatty acid composition of the dietary oils is presented in Table 3.2. All oils contained the following antioxidants (in mg/kg): mono-tertbutyl hydroquinine (200), citric acid (200) and 1,2-dihydro-6-ethoxy-2,4,4-trimethylquinoline (EMQ, 100). Moreover, the following antioxidants were added to the complete diets: EMQ (150), α -tocopherol acetate (150) and NaHSeO₃ (0.38).

Table 3.1: Composition of Experimental Diets

Ingredients	Energy (g.4184 kJ ⁻¹)			Energy %
	Diet			
	MO	SO	HCO	
Casein	62.0	62.0	62.0	23
Minerals ^{a)}	5.4	5.4	5.4	-
Vitamin mix ^{b)}	1.0	1.0	1.0	-
Cellulose	15.0	15.0	15.0	-
Sucrose	65.8	65.8	65.8	27
Mackerel oil (MO)	48.4	-	-	
Sunflowerseed oil (SO)	5.4	53.8	5.4	50
Hydrogenated coconut oil (HCO)	-	-	48.4	

a) g.4184 kJ⁻¹: KCl 0.350, MgHPO₄·3H₂O 0.956, KH₂PO₄ 0.475, KHCO₃ 0.719, C₆H₅Na₃O₇·2H₂O 0.711, CaCO₃ 2.014, MnC₆H₅O₇ 0.0756, C₆H₅FeO₇·5H₂O 0.0439, Cu₂C₆H₆O₈ 0.0036, Zn₃(C₆H₅O₇)₂·2H₂O 0.0125, KIO₃ 0.00007

b) g.4184 kJ⁻¹: choline 0.250, vitamin E 0.020, calciumsilicate 0.05, myoinositol 0.025, vitamin B₁₂ 0.000005, vitamin A 0.0077, niacin 0.005, pantothenic acid 0.005, riboflavin 0.0015, thiamin 0.0015, vitamin D 0.00125, vitamin K 0.000227, vitamin B₆ 0.0005, folic acid 0.00025, biotin 0.00005, made up to 1.00 with sucrose

Chemicals

Metrizamide® was purchased from Nyegaard & Co. (Oslo, Norway). Heparin (Thromboliquine®) was obtained from Organon Teknika (Boxtel, the Netherlands). Gelatin, LiChroprep C8 (40 µm particles), LiChroprep C18 (40 µm particles), Kieselgel 60 (7-230 mesh) and other reagents (all analytical grade) were purchased from Merck (Darmstadt, FRG). Hexane, chloroform, methanol, dichloromethane, diethyl ether, acetonitrile, tetrahydrofuran and ethyl acetate (all HPLC-grade) were from Rathburn (Walkerburn, United Kingdom). Triethylamine (99%) and methoxylamine hydrochloride were obtained from Janssen Chimica (Tilburg, the Netherlands). Alumina N grade super I was purchased from ICN Medicals (Amsterdam, the Netherlands). Panacylbromide (p-(9-anthroyloxy)phenacylbromide) was from Molecular Probes Inc. (Eugene, USA). Water was purified with a milli-Q purification unit from Millipore (Bedford, USA).

Mast cell Isolation and Activation

Mast cells were obtained by peritoneal lavage as described in Chapter 2. After purification, these cells were resuspended in buffer pH 7.4, consisting of 136 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄ and 0.1% gelatin (w/v), to a final concentration 10⁶ cells/ml. The cells were activated with 10 µM of the calcium ionophore A23187 at 37 °C in the presence of 2 mM CaCl₂. After 20 min, the mixture was cooled, centrifuged at 195 x g for 5 min (4 °C) and supernatants were collected. To the supernatant, 1 ml of methanol was added and samples were stored at -80 °C until analysis.

Table 3.2: *Fatty Acid Composition of the Dietary Oils (% of total Fatty Acids)^{a)}*

Fatty Acid	Diet		
	MO ^{b)}	SO	HCO
8:0			3.6
10:0			8.6
12:0			38.9
14:0	5.4		17.5
16:0	10.7	6.4	10.2
18:0	2.3	4.5	12.2
20:0	0.1	0.4	0.1
22:0		0.1	
24:0		0.2	
16:2(n-4)	0.3		
16:1(n-7)	2.8	0.2	
18:1(n-7)/(n-9)	14.3	15.3	1.5
20:1(n-9)/(n-11)	9.9	0.3	0.1
22:1(n-9)/(n-11)	15.2	0.8	0.1
24:1(n-9)	0.4		
18:2(n-6)	8.4	70.8	7.1
18:3(n-6)	0.3		
20:2(n-6)	0.3		
20:3(n-6)	0.1		
20:4(n-6) + 20:3(n-3)	0.3		
22:4(n-6)	0.6		
18:3(n-3)	1.2	0.3	
18:4(n-3)	4.2		
20:4(n-3)	1.0		
20:5(n-3)	7.3		
22:5(n-3)	1.2		
22:6(n-3)	12.9		

^{a)} Based on the fatty acid composition of the oils (MO: mackerel oil; SO: sunflowerseed oil and HCO: hydrogenated coconut oil) and corrected for the proportions of the oil in the diets (see Table 3.1)

^{b)} Determined by the supplier of the oil: the Norwegian Herring Oil and Meal Industries (Dr. J. Opstvedt), Fijllingdalen, Norway.
When no data are given, the amounts were below detection limits.

Lipid Extraction and Fatty Acid Analysis

Purified peritoneal mast cells were resuspended in 1 ml of buffer pH 7.4 to a final concentration of $7 \cdot 10^5$ cells/ml. To this suspension, 1 ml of methanol was added. To all organic solvents used the anti-oxidant 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT, 0.005% w/v) was added to prevent oxidation of the fatty acids. Mast cell phospholipid fatty acids were determined according to Roemen *et al.*¹⁶ Briefly, lipids were extracted according to Folch *et al.*⁶ Total phospholipids were isolated from other lipids by thin layer chromatography after pre-developing the plates with chloroform/methanol 1:1 (v/v).

After application of the sample, plates were developed with chloroform/methanol/acetic acid/water 10/10/1/1 (v/v) followed by petroleum ether/diethyl ether/acetic acid 120/25/1.5 (v/v). Phospholipids were visualized by spraying with a 0.01% rhodamine solution in methanol. Subsequently phospholipids were hydrolyzed and their fatty acids methylated using 14% borontrifluoride BF_3 (w/v) in methanol at 100 °C for one hour. The methyl ester mixtures were analyzed using a gaschromatograph (Perkin Elmer, Autosystem) fitted with a WCOT fused-silica capillary column coated with 0.12 μm CP-Sil 5 CB (50 m x 0.25 mm I.D., Chrompack International, Middelburg, the Netherlands). The temperature of the injector and the flame-ionization detector were set at 300 °C. The starting temperature of the column was 160 °C. Thereafter, temperature of the column consecutively increased by 3°C/min to 229 °C, by 2 °C/min to 275 °C and by 5 °C to 290 °C. Routinely, 2 μl of the methyl ester mixture was injected. Using the split injection technique, 1/37 of this volume was applied to the column. Carrier gas was helium (140 kPa). Peak areas were determined with PE Nelson Turbochrom II. 1,2 dinonadecanoyl-sn-glycero-3-phosphocholine (PC 19:0) was added to the samples before lipid-extraction in order to calculate the quantitative amounts. Quantifying the amount of methyl esters was based on the ratio between the peak area 19:0 and the peak area of the fatty acid methyl esters in the samples multiplied by the amount of 19:0 present in the standard solution. Results are expressed as % of total fatty acids.

Determination of Prostanoids and Hydroxy Fatty Acids

Prostanoids were determined according to Engels *et al.*⁵ Briefly, after addition of $\text{PGF}_{1\alpha}$ and PGE_1 as internal standards, chloroform/methanol extraction and purification on silica columns, samples were methoximated with methoxylamine hydrochloride. After purification on C8 reversed-phase material and esterification with panacylbromide, the panacylesters were separated and analyzed by C18 reversed-phase high performance liquid chromatography (HPLC) with fluorometric detection. Hydroxy fatty acids and HHT were determined according to Claeys *et al.*⁴ by C18 reversed-phase HPLC with ultraviolet detection at 235 nm. 15-hydroxy-11,13-eicosadienoic acid, prepared from 10,14 *cis*-eicosadienoic acid⁴ was used as internal standard to calculate the quantitative amounts. Results are expressed as pmol/ 10^6 cells per 20 min.

Statistical Analysis

Data are mean values $\pm$ SEM. Columns with different superscripts differ significantly from one another ($P \leq 0.05$). Comparisons were made using an analysis of variance followed by Tukey's method to evaluate the effect of changes in the dietary fat type.²⁰

3.4 RESULTS

Rat Growth

No differences in body weights and relative weight gains of rats on different diets were observed over the feeding period. Body weight (in gram, mean $\pm$ SD) before the

feeding period were: MO, 200 ± 25; SO, 205 ± 20 and HCO, 215 ± 23. At the end of the feeding period weights were: MO, 430 ± 30; SO, 435 ± 27 and HCO, 452 ± 32.

Fatty Acid Composition of Mast Cell Phospholipids

The total amount of mast cell phospholipid fatty acids attributed to 14.20 ± 0.94, 14.75 ± 0.75 and 14.67 ± 0.97 µg/10⁶ cells in rats fed MO, SO or HCO, respectively. No significant differences were observed between the three dietary groups. The high amount of stearic acid, 18:0, in the saturated fat-diet (HCO) was accompanied by a significant increase of this fatty acid in mast cell phospholipids (Table 3.3).

Table 3.3: Mast cell Phospholipid Fatty Acid Composition (% of total Fatty Acids)

Fatty Acid	Diet		
	MO	SO	HCO
14:0	1.0 ± 0.3	0.4 ± 0.3	1.4 ± 0.2
16:0	29.6 ± 1.0 ^a	23.7 ± 0.9 ^b	21.7 ± 1.0 ^b
18:0	20.6 ± 0.8 ^a	23.0 ± 1.2 ^a	27.9 ± 1.0 ^b
20:0		0.8 ± 0.4 ^a	1.3 ± 0.3 ^a
22:0			0.7 ± 0.3
24:0			0.6 ± 0.3
18:1(n-7)	4.0 ± 0.1 ^a	1.4 ± 0.4 ^b	1.9 ± 0.3 ^b
18:1(n-9)	11.0 ± 0.2	12.1 ± 0.9	9.1 ± 1.1
24:1(n-9)	2.7 ± 0.4	2.6 ± 0.4	2.0 ± 0.4
18:2(n-6)	7.2 ± 0.7 ^a	10.1 ± 0.6 ^b	5.8 ± 0.3 ^a
20:2(n-6)		1.7 ± 0.5 ^a	0.2 ± 0.1 ^b
20:4(n-6)	8.8 ± 0.3 ^a	14.9 ± 1.0 ^b	19.5 ± 1.1 ^c
22:4(n-6)		6.6 ± 0.5 ^a	6.9 ± 0.4 ^a
22:5(n-6)			0.3 ± 0.2
24:2(n-6)		2.1 ± 0.4 ^a	
20:5(n-3)	5.4 ± 0.3 ^a		
22:5(n-3)	5.8 ± 0.6 ^a		
22:6(n-3)	2.9 ± 0.6 ^a		

Peritoneal mast cells were isolated from rats fed diets differing in their fatty acid composition. MO: mackerel oil, SO: sunflowerseed oil and HCO: hydrogenated coconut oil. Phospholipid fatty acid methyl esters (n=6 per group) were prepared and analyzed as described in Materials and Methods. Values in rows with different superscripts differ significantly from each other (P ≤0.05).

Similarly, the high content of linoleic acid, 18:2(n-6), in the SO-diet resulted in an increased content of this fatty acid in mast cell phospholipids. However, this was not accompanied by an increased content of arachidonic acid, 20:4(n-6), in mast cell phospholipids. The high contents of timnodonic acid, 20:5(n-3), clupanodonic acid, 22:5(n-3), and cervonic acid, 22:6(n-3), in the MO-diet were reflected by the presence of relatively high amounts of these fatty acids in mast cell phospholipids. The presence of (n-3) fatty acids was associated with a significant decrease in the contents of arachidonic acid, 20:4(n-6), and adrenic acid, 22:4(n-6), one of its elongation products.

The arachidonic acid content was highest in mast cell phospholipids from the HCO-group (Table 3.3).

Release of Prostanoids and Hydroxy Fatty Acids

The results showed that, irrespective of the diet, major products released by A23187-stimulated rat peritoneal mast cells were PGD₂, PGE₂, HHT, 9-HODE and 13-HODE (Table 3.4). Minor amounts of 6-keto-PGF₁α, PGF₂α, TxB₂, 15-HETE and 11-HETE were also released. Preliminary studies, in which the mast cell pellet was extracted with alcohol, showed amounts of prostanoids and hydroxy fatty acids which were about equal to the detection limits, indicating that these compounds, once produced, were rapidly released from the cells.

Table 3.4: *Metabolites of Linoleic Acid (LA) and Arachidonic Acid (AA) produced by Activated Peritoneal Mast Cells from Rats fed different Diets*

Compounds	Diet		
	MO	SO	HCO
AA-Metabolites			
6-keto-PGF ₁ α	1.7 ± 0.9	4.4 ± 1.3	4.0 ± 1.0
PGF ₂ α	0.9 ± 0.6	2.8 ± 2.5	4.6 ± 2.8
TxB ₂	2.3 ± 1.5	2.1 ± 1.0	4.2 ± 2.9
PGD ₂	31.9 ± 8.5 ^a	109.3 ± 17.3 ^b	136.9 ± 16.9 ^b
PGE ₂	5.7 ± 1.8 ^a	19.6 ± 2.7 ^b	25.4 ± 8.2 ^b
HHT	33.0 ± 5.0	47.5 ± 9.1	53.9 ± 6.9
15-HETE	5.1 ± 2.4	5.5 ± 5.5	8.4 ± 2.2
11-HETE	6.6 ± 2.9	10.0 ± 4.5	13.0 ± 3.6
LA-Metabolites			
9-HODE	22.6 ± 3.3 ^a	104.7 ± 9.4 ^b	51.9 ± 5.1 ^c
13-HODE	29.7 ± 3.8 ^a	152.9 ± 20.7 ^b	75.5 ± 6.4 ^c

Peritoneal mast cells were isolated from rats fed diets differing in their fatty acid composition. Mast cells (n=6 per group) were incubated in the presence of 2 mM CaCl₂ and were stimulated with 10 μM calcium ionophore A23187 at 37 °C for 20 min. The products were determined in the supernatants as described in Materials and Methods. Within each row, columns with different superscripts differ significantly from each other (P ≤ 0.05). Results are expressed as pmol/10⁶ cells per 20 min. MO: mackerel oil, SO: sunflowerseed oil and HCO: hydrogenated coconut oil.

Upon activation, in the group with the highest linoleic acid content in mast cell phospholipids (SO-group), the formation of 9-HODE and 13-HODE, cyclooxygenase and lipoxygenase products from linoleic acid, respectively, were highest. The production of linoleic acid-derived 9-HODE and 13-HODE were lowest in mast cell phospholipids from the MO-group, although the lowest linoleic acid values were observed in mast cell phospholipids of the HCO-group. The relatively low arachidonic acid content in mast cell phospholipids from the MO-group was accompanied by a moderate release of PGD₂ and PGE₂ upon activation. When compared to this group, the higher content of arachidonic acid in the SO- and HCO-group was accompanied by an increased

production of PGD_2 and PGE_2 upon activation. Although not statistically different, similar trends were observed for other cyclooxygenase products from arachidonic acid, such as 6-keto $\text{PGF}_{1\alpha}$, $\text{PGF}_{2\alpha}$, TxB_2 , HHT and lipoxygenase products such as 11-HETE and 15-HETE. The production of 12-HEPE, a lipoxygenase product from timnodonic acid, could not be detected.

3.5 DISCUSSION

We studied the effects of diet-induced changes in mast cell phospholipid fatty acid composition on the release of prostanoids and hydroxy fatty acids. To this end, peritoneal mast cells, isolated from rats fed diets differing in their fatty acid composition, were stimulated with the calcium ionophore A23187. Timnodonic acid, 20:5(n-3), the precursor of the prostanoids of the 3-series, clupanodonic acid, 22:5(n-3), and cervonic acid, 22:6(n-3), which were present in considerable amounts in the MO-diet, were readily incorporated in mast cell phospholipids from the MO-group. This was accompanied by a significant decrease in (n-6) fatty acids, such as arachidonic acid, 20:4(n-6), and one of its elongation products, 22:4(n-6) in mast cell phospholipids. It was found that the presence of large amounts of saturated fatty acids in the diet (HCO-group) resulted in the incorporation of high amounts of arachidonic acid in mast cell phospholipids. Similar effects have been found in rat platelets and cultured human umbilical vein endothelial cells.^{9,19} The increased arachidonic acid content is most probably due to the high dietary intake of saturated acids, which is known to stimulate $\Delta 6$ -desaturase.² It was also found that the high content of linoleic acid in the SO-diet resulted in an increased incorporation of this fatty acid in mast cell phospholipids. This was not accompanied by high levels of arachidonic acid, which is most probably due the fact that high contents of dietary linoleic acid result in inhibition of $\Delta 6$ -desaturase (substrate-inhibition).¹²

In general, a linear relation was observed between the arachidonic acid (AA) content in mast cell phospholipids (PL) and the formation of prostanoids and hydroxy fatty acids by these cells (Fig. 3.1A). For instance, the formation of PGD_2 and PGE_2 was high when the arachidonic acid content in membrane phospholipids was high (HCO-group), whereas the formation of these compounds was low when the arachidonic acid content was low (MO-group). Although not statistically significant, similar trends were observed for other cyclooxygenase and/or lipoxygenase products from arachidonic acid, such as $\text{PGF}_{2\alpha}$, HHT and 11-HETE. These results strongly suggest that in mast cells, the arachidonic acid content in membrane phospholipids is an important determinant in the production of prostanoids and hydroxy fatty acids derived from this fatty acid. Similar conclusions could be reached for the linoleic acid content in membrane phospholipids and the formation of 9-HODE and 13-HODE, cyclooxygenase and lipoxygenase products from linoleic acid¹⁷, respectively. It should be noted that in order to obtain a linear relation between the linoleic acid content in membrane phospholipids and the formation of these hydroxy fatty acids, it was assumed that (n-3) polyunsaturated fatty acids (PUFA) were about 3 times as effectively converted by cyclooxygenase and lipoxygenase enzyme systems as linoleic acid (LA). This is based on the well-known

finding that the affinity of cyclooxygenase and lipoxygenase for linoleic acid is much lower than for (n-3) fatty acids.¹¹

Figure 3.1: Formation of prostanoids and hydroxy fatty acids in mast cells: relation to availability of precursor fatty acids. AA: arachidonic acid; FA: fatty acid; PL: phospholipid; LA: linoleic acid; PUFA: polyunsaturated fatty acid

9-HODE and 13-HODE are formed by various cells such as endothelial cells^{3,8} and neutrophils.^{4,8} Results from Henricks *et al.*¹⁰ indicate that 13-HODE, the predominant lipoxygenase product from linoleic acid in mast cells, may play a role in inflammatory reactions, because of its chemotactic activity on polymorphonuclear leukocytes. PGD₂, the main cyclooxygenase product from arachidonic acid in mast cells, has been shown to potentiate histamine-induced increase of endothelial permeability.^{15,18} Therefore, it is tempting to speculate that diet-induced changes in the production of prostanoids and hydroxy fatty acids by mast cells, may ultimately have consequences for processes such as the inflammatory response, in which these mast cell-derived products are putatively involved.

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| CHAPTER 4

Histamine Release by Compound 48/80-Stimulated Rat Peritoneal Mast Cells: Role of the Dietary Fat Type

4.1 ABSTRACT

Previously, it was found that the fatty acid composition of mast cell phospholipids and the formation of prostanoids and hydroxy fatty acids by these cells was significantly influenced by the dietary fatty acid composition. In the present study, we determined to which extent the degranulation process in mast cells was related to the fatty acid composition of membrane phospholipids. To this end, peritoneal mast cells were isolated from rats fed diets which differed in their fatty acid compositions: (i) genuine salmon oil (GSO), abundant in (n-3) fatty acids, (ii) sunflowerseed oil (SO), rich in (n-6) fatty acids, particularly linoleic acid, and (iii) hydrogenated coconut oil (HCO), rich in saturated fatty acids. Mast cells were stimulated with varying concentrations of the mast cell-degranulating agent compound 48/80. The extent of mast cell degranulation was quantified by determination of histamine in the supernatants. No differences in compound 48/80-induced histamine release between the three dietary groups for any of the concentrations of compound 48/80 tested were found. This finding strongly suggests that the extent of mast cell degranulation is independent of the fatty acid composition of membrane phospholipids.

4.2 INTRODUCTION

Biomembranes are composed of proteins, cholesterol and a mixture of phospholipids. Fatty acids, as part of membrane phospholipids, may serve as substrates for the cyclooxygenase and/or lipoxygenase enzyme systems. It has been found that changes in the dietary fat type significantly influenced the fatty acid composition of mast cell phospholipids and the formation of prostanoids and hydroxy fatty acids (*Chapter 3*). In rat platelets, similar to mast cells, there is a strict relationship between the arachidonate level in membrane phospholipids and thromboxane A₂ formation.⁷ In addition, it has been shown that the sensitivity of platelets to agonists, such as thromboxane, is significantly influenced by the type of dietary fat, *i.e.*, platelets from rats fed diets rich in linoleic acid (SO-diet), are more sensitive to the aggregating effect of a low dose of collagen as compared to platelets of other diets.⁵ Platelets from rats fed high amounts of saturated fatty acids (HCO-diet) have been described to show a decreased responsiveness in terms of Ca²⁺ signaling, secretion and aggregation with agonists such as the thromboxane A₂ analogue U46619 and thrombin, when compared to platelets from rats fed other diets.⁴ In this process, the membrane cholesterol/phospholipid ratio plays a prominent role.⁴ Results in human platelets have suggested that incorporation of docosahexaenoic acid, 22:6(n-3) and, to a lesser degree, eicosapentaenoic acid, 20:5(n-3) into platelet membranes suppress thromboxane A₂ receptor function and thromboxane-mediated responses.^{1,8} Similar conclusions have been reached by other authors using a different experimental approach.⁹ Thus, besides its influence on the formation of eicosanoids, changes in the dietary fat type may also influence specific cellular processes and functions.

We studied to which extent in mast cells the degranulation process was influenced by diet-induced changes in membrane fatty acid composition. Therefore, rats were fed

diets comparable to those described in Chapter 3, *i.e.*, genuine salmon oil (GSO), rich in (n-3) fatty acids, sunflowerseed oil (SO), rich in linoleic acid, 18:2(n-6), and hydrogenated coconut oil (HCO), rich in saturated fatty acids. Peritoneal mast cells were isolated and incubated with varying concentrations of the G protein-activating compound 48/80.

Although changes in the dietary fatty acid composition resulted in significant changes in the fatty acid composition of mast cell phospholipids, no significant differences in compound 48/80-induced histamine release were observed between the three dietary groups. The results strongly suggest that in mast cells, the extent of degranulation was independent of the fatty acid composition of membrane phospholipids.

4.3 MATERIALS AND METHODS

Animals

Adult Wistar rats were obtained from a specified pathogen-free colony of the Charles River Laboratory (Sulzfeld, FRG). All animals were kept in stainless steel cages (1 rat per cage) and had free access to food and water. Experiments were performed according to institutional guidelines.

Diets

Animals were randomly divided into 3 groups and were fed, *ad libitum*, adequate diets containing 50 energy% (en%) fat (see Table 3.1), but which differed in their fatty acid compositions:

1. *GSO-diet*: 45 en% mackerel oil (GSO) (supplier: Norwegian Herring Oil and Meal Industries, Fijllingdalen, Norway), rich in long-chain (n-3) polyunsaturated fatty acids, 20:5(n-3) and 22:6(n-3) in particular, supplemented with 5 en% sunflowerseed oil (SO) to ensure an adequate supply of (n-6) essential fatty acids
2. *SO-diet*: 50 en% sunflowerseed oil, rich in (n-6) fatty acids, 18:2(n-6) in particular
3. *HCO-diet*: 45 en% hydrogenated coconut oil (HCO), rich in saturated fatty acids, supplemented with 5 en% SO.

The relative fatty acid composition of the diets is presented in Table 4.1. The diets were freshly prepared once a week and were stored at -20 °C. Other conditions were similar to those described in Chapter 3.

Mast cell Activation

Mast cells were obtained by peritoneal lavage as described in Chapter 2. After purification, mast cells were resuspended in buffer pH 7.4, consisting of (in mM): NaCl (136), KCl (2.7), Hepes (10), glucose (5.0) and gelatin (0.1%, w/v) to a final concentration of 10⁶ cells/ml. One rat was used for each experiment. Samples of 125 µl were brought into 875 µl of buffer pH 7.4 and were preincubated at 37 °C for 5 min. Mast cell

degranulation was evoked by addition of varying concentrations of compound 48/80 (final concentrations 0, 0.1, 0.75, 1.5, 3, 5 and 10 $\mu\text{g/ml}$). Incubations were carried out in the presence of 1 mM CaCl_2 . Total releasable histamine was determined upon incubation of the cells with Triton X-100 (0.1%, w/v). After incubation for 5 min in the presence of compound 48/80, 500 μl samples were withdrawn and immediately layered onto 250 μl of silicon oil. After centrifugation at 15,000 $\times g$ for 20 s, supernatants were collected and 1-methyl histamine was added as internal standard. Samples were stored at -80°C until analysis.

Compound 48/80-induced histamine release was expressed as percentage of total mast cell histamine after correction for blank histamine release (*i.e.*, histamine release in the absence of agonist), which was $<5\%$ in all experiments.

Table 4.1 *Fatty Acid Composition of the Diets (% of Total Fatty Acids)*

Fatty Acid	Diet		
	GSO	SO	HCO
10:0	0.4		5.0
12:0		0.1	42.7
14:0	3.5	0.1	16.8
15:0	0.4		
16:0	14.2	6.3	11.1
17:0	0.2		
18:0	3.6	4.6	12.9
20:0		0.3	0.2
22:0		0.7	0.1
24:0		0.2	0.1
16:1(n-7)	7.1	0.1	
18:1(n-7)tr	1.5		
18:1(n-7)	3.1	0.6	0.1
18:1(n-9)	17.0	24.8	3.6
18:2(n-9)		0.4	0.1
20:1(n-9)	4.8	0.2	0.1
22:1(n-9)	1.0		
24:1(n-9)	0.5		
18:2(n-6)	9.8	60.9	7.1
20:2(n-6)	0.7		
22:4(n-6)	0.7		
18:3(n-3)	1.4		
18:4(n-3)	2.0		
20:5(n-3)	10.5		
22:5(n-3)	4.4		
22:6(n-3)	9.3		

Methyl esters of dietary fatty acids were prepared and analyzed as described in Materials and Methods. Amounts are presented as % of total fatty acids. GSO: genuine salmon oil; SO: sunflowerseed oil; HCO: hydrogenated coconut oil.

Fatty Acid Composition of Mast Cell Phospholipids

After addition of the internal standard (consisting of 62 mg PC 19:0 and 100 mg 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) dissolved in 100 ml of methanol), total lipid extraction was performed according to Bligh and Dyer.² Aminopropyl bonded silica columns (500 mg) were used to separate phospholipids from the total lipid extract.⁶ Phospholipids were hydrolyzed and their fatty acids methylated using 14% borontrifluoride BF_3 (w/v) in methanol. Samples were incubated at 100 °C for one hour. Methyl ester mixtures were separated and quantified using a gaschromatograph (Perkin Elmer, Autosystem) fitted with a 50 m CP-Sil 88 polar capillary column with 0.25 mm I.D and 0.20 μm film thickness (Chrompack, Middelburg, the Netherlands). Routinely, 2 μl of the mixture was injected. Split ratio was 1:20. The temperature of the injector and the detector were set at 300 °C. Starting temperature of the column was 160 °C. After 10 min, the temperature increased up to 190 °C with a rate of 2.5 °C/min and finally after 20 min increased to 230 °C with a rate of 4 °C/min. This temperature was kept constant for another 10 min. Carrier gas was helium (140 kPa). Peak areas were determined using the Turbochrom II integration system. A standard methyl ester mixture was used to identify the fatty acid methyl esters by means of the retention times. Quantification of the amount of methyl esters was based on the ratio between the peak area 19:0 and the peak area of the fatty acid methyl esters in the samples, multiplied by the amount of 19:0 present in the standard solution. Results are expressed as % of total fatty acids.

Determination of Histamine

Histamine was used to quantitate the extent of mast cell degranulation. This compound was determined in supernatants of stimulated cells by High-Performance Liquid Chromatography as described in Chapter 2.

Statistical Analysis

Data are presented as mean values $\pm$ SEM. Columns with different superscripts differ significantly from each other ($P \leq 0.05$). Comparisons were made using an analysis of variance followed by Tukey's method for multiple comparisons to evaluate the effect of changes in the dietary fat type.¹³

4.4 RESULTS

Fatty Acid Composition Mast Cell Phospholipids

In general, diet-induced changes in the fatty acid composition of mast cell phospholipids were comparable to those observed earlier (*Chapter 3*). For instance, the high content of (n-3) fatty acids in the fish-oil diet (GSO-group), resulted in an increased incorporation of timnodonic acid, 20:5(n-3), clupanodonic acid, 22:5(n-3), and cervonic acid, 22:6(n-3), in mast cell phospholipids (Table 4.2). The increased content of (n-3) fatty acids was accompanied by decreased contents of (n-6) fatty acids such as arachidonic acid, 20:4(n-6), and one of its elongation products, adrenic acid, 22:4(n-6). Again, the arachidonic acid content in mast cell phospholipids was highest in the HCO-

group. When compared to the other two dietary groups, the high content of linoleic acid (LA), 18:2(n-6), in the SO-diet was accompanied by an increased content of this fatty acid and 22:2(n-6), a LA-elongation product, in mast cell phospholipids.

Table 4.2: Fatty Acid Composition of Mast Cell Phospholipids (% of total Fatty Acids)

Fatty Acid	Diet		
	GSO	SO	HCO
10:0	2.5 ± 0.1	1.9 ± 0.2	1.6 ± 0.4
12:0	1.7 ± 0.1 ^a	1.4 ± 0.1 ^{ab}	1.2 ± 0.1 ^b
15:0	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
16:0	24.3 ± 0.2 ^a	21.7 ± 0.5 ^b	20.7 ± 0.2 ^b
17:0	1.4 ± 0.1 ^a	1.1 ± 0.1 ^b	0.9 ± 0.1 ^b
18:0	23.2 ± 0.6 ^a	26.4 ± 0.4 ^b	26.8 ± 1.0 ^b
20:0	1.3 ± 0.1	1.2 ± 0.2	1.8 ± 0.1
21:0	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
22:0	0.4 ± 0.1 ^a	0.5 ± 0.1 ^a	1.2 ± 0.1 ^b
23:0	0.5 ± 0.2 ^a		
24:0	0.5 ± 0.1 ^a	0.6 ± 0.1 ^a	1.3 ± 0.1 ^b
16:1(n-7)tr	0.9 ± 0.1 ^a	0.8 ± 0.1 ^{ab}	0.5 ± 0.1 ^b
16:1(n-7)	0.7 ± 0.1 ^a	0.3 ± 0.1 ^b	0.4 ± 0.1 ^b
18:1(n-7)tr	0.9 ± 0.1	0.6 ± 0.3	0.2 ± 0.1
18:1(n-7)	1.9 ± 0.1 ^a	0.9 ± 0.1 ^b	1.4 ± 0.1 ^c
18:1(n-9)	7.4 ± 0.5	7.7 ± 0.4	6.7 ± 0.1
20:1(n-9)tr	0.4 ± 0.1	0.7 ± 0.5	
20:1(n-9)	2.0 ± 0.1 ^a	1.1 ± 0.1 ^b	0.8 ± 0.1 ^c
24:1(n-9)	4.8 ± 0.3 ^a	2.2 ± 0.1 ^b	2.7 ± 0.5 ^b
18:2(n-6)	5.1 ± 0.3 ^a	7.1 ± 0.2 ^b	4.7 ± 0.3 ^a
20:2(n-6)	0.3 ± 0.1 ^a	0.8 ± 0.1 ^b	0.4 ± 0.1 ^b
20:3(n-6)	1.7 ± 0.7	1.2 ± 0.1	1.6 ± 0.1
20:4(n-6)	5.0 ± 0.2 ^a	12.1 ± 0.5 ^b	15.0 ± 0.5 ^c
22:2(n-6)	0.3 ± 0.1 ^a	0.5 ± 0.1 ^b	0.2 ± 0.1 ^a
22:4(n-6)	0.6 ± 0.1 ^a	4.3 ± 0.6 ^b	4.9 ± 0.3 ^b
22:5(n-6)	0.4 ± 0.1 ^a	0.5 ± 0.1 ^{ab}	0.7 ± 0.1 ^b
24:2(n-6)	0.3 ± 0.1 ^a	1.3 ± 0.1 ^b	0.8 ± 0.1 ^c
18:3(n-3)	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
20:5(n-3)	3.4 ± 0.2 ^a	0.2 ± 0.1 ^b	0.2 ± 0.1 ^b
22:5(n-3)	4.2 ± 0.1 ^a	0.5 ± 0.1 ^b	0.6 ± 0.1 ^b
22:6(n-3)	2.6 ± 0.1 ^a	1.4 ± 0.3 ^b	1.2 ± 0.1 ^b

Peritoneal mast cells were isolated from rats fed diets differing in their fatty acid composition. GSO: genuine salmon oil; SO: sunflowerseed oil; HCO: hydrogenated coconut oil. Methyl esters of mast cell phospholipid fatty acids were prepared and analyzed as described in Materials and Methods. Amounts, expressed as % of total fatty acids, are presented as mean values ± SEM (GSO and HCO, n=4 per group, SO, n=5 per group). Within each row, columns with different superscripts differ significantly from each other ($P \leq 0.05$).

Mast Cell Degranulation

Mast cells were incubated with varying concentrations of compound 48/80 in the presence of 1 mM CaCl_2 . At concentrations of compound 48/80 of 0.1 or 0.25 $\mu\text{g/ml}$, histamine release was negligible, *i.e.*, <5% of the total mast cell histamine content (Fig. 4.1). At a concentration of 0.5 $\mu\text{g/ml}$, histamine release significantly increased, but no significant differences were observed between the three dietary groups ($37 \pm 3\%$, $43 \pm 3\%$ and $36 \pm 5\%$ in the GSO, HCO and SO-group, respectively). At a concentration of 1.5 $\mu\text{g/ml}$, compound 48/80-induced histamine release in the three groups was sub-maximal, reaching values of $64 \pm 3\%$, $67 \pm 4\%$ and $69 \pm 5\%$, respectively. Histamine release was about maximal at a concentration of 10 $\mu\text{g/ml}$, reaching values of $73 \pm 4\%$, $75 \pm 5\%$ and $78 \pm 4\%$, respectively. No additional increases in compound 48/80-induced histamine release were observed at concentrations higher than 10 $\mu\text{g/ml}$ (*data not shown*). No significant differences in histamine release were observed between the three dietary groups for any of the concentrations of compound 48/80 tested.

Figure 4.1: Compound 48/80-induced histamine release

Mast cells, isolated from rats fed different diets, genuine salmon oil, GSO (●), hydrogenated coconut oil, HCO (■) or sunflowerseed oil, SO (◆) were activated with various concentrations of the mast cell degranulating agent compound 48/80 in the presence of 1 mM CaCl_2 . Histamine release (mean values $\pm$ SEM, $n=10$) is expressed as % of total histamine content after correction for histamine release in the absence of agonist.

4.5 DISCUSSION

Diet-induced changes in the fatty acid composition of mast cell phospholipids were comparable to those observed before (*Chapter 3*). For instance, the introduction (n-3) fatty acids in the diet (MO-group) resulted in an increased incorporation of timnodonic acid, 20:5(n-3), clupanodonic acid, 22:5(n-3), and cervonic acid, 22:6(n-3), in mast cell phospholipids. This was accompanied by a significant decrease in the contents of (n-6) fatty acids, such as arachidonic acid (AA), 20:4(n-6), and one of its elongation products, adrenic acid, 22:4(n-6). In agreement with earlier results, it was found that the arachidonic acid content was highest in mast cell phospholipids in the HCO-group, a phenomenon which is most probably due to the stimulation of $\Delta 6$ -desaturase by the high content of saturated fatty acids.³ Again, the content of linoleic acid, 18:2(n-6), in mast cell phospholipids was highest when the content of this fatty acid was high, *i.e.*, in the SO-group. The results strengthen our earlier notion that the fatty acid composition of mast cell phospholipids is partially determined by the fatty acid composition of the diet.

Despite considerable changes in the fatty acid composition of mast cell phospholipids, no significant differences in compound 48/80-induced histamine release were observed between the three dietary groups. These results indicate that the extent of mast cell degranulation is not related to the fatty acid composition of membrane phospholipids. Previously, we have shown that the formation of prostanoids and hydroxy fatty acids was closely related to the fatty acid composition of membrane phospholipids (*Chapter 3*). Therefore, the present results also indicate that the extent of mast cell degranulation is not related to the formation of prostanoids and hydroxy fatty acids. This will be discussed in detail in Chapter 5.

Experiments with cultured human umbilical vein endothelial cells (HUVEC) have shown that the fatty acid composition of membrane phospholipids can extensively be modified by the addition of specific polyunsaturated fatty acids (PUFA). The decreased arachidonic acid content evoked by long-term incubation of HUVEC in the presence of (n-3) fatty acids was accompanied by a decreased formation of eicosanoids, such as 6-keto-PGF₁ α or PGF₂ α , when these cells were stimulated with thrombin.¹² In addition, the sensitivity of isolated endothelial phospholipids to peroxidation has been found to be directly proportional to the number of double bonds present.^{11,12} In contrast, no effects of fatty acid modification in various membrane-related processes, such as the adherence of polymorphonuclear leukocytes and monocytes to the endothelial cell monolayer or the facilitation of fibrin-clot formation in recalcified human plasma (*i.e.*, procoagulant activity) were observed.¹⁰ It was concluded that, in general, the fatty acid composition of endothelial phospholipids could extensively be changed without influencing cellular function. Only in those reactions in which the fatty acids *as such* were involved, such as in the formation of eicosanoids and in lipid peroxidation, changes in the membrane fatty acid composition appeared to play an important role. Our findings in mast cells suggest that also in those cases in which fatty acids, as part of membrane phospholipids, mainly serve to create a suitable microenvironment for membrane-related processes, such as in the exocytotic process, diet-induced changes

in membrane phospholipid fatty acid composition do not play an important role. Conversely, in those cases in which fatty acids actively participate in cellular processes, such as in the formation of prostanoids and hydroxy fatty acids, diet-induced changes in mast cell phospholipid fatty acid composition appears to play a prominent role.

In conclusion, despite considerable changes in mast cell phospholipid fatty acid composition, no significant differences in compound 48/80-induced histamine release were observed between the three dietary groups. The results strongly suggest that the extent of mast degranulation is not related to the fatty acid composition of membrane phospholipids.

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CHAPTER 5

Differential Release of Histamine and Prostaglandin D₂ in Rat Peritoneal Mast Cells: Roles of Cytosolic Calcium and Protein Tyrosine Kinases

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Charles M.C.J. van Haaster, Wim Engels, Paul J.M.R. Lemmens, Gerard Hornstra, Ger J. Van der Vusse and Johan W.M. Heemskerk

Differential release of histamine and prostaglandin D₂ in rat peritoneal mast cells: roles of cytosolic calcium and protein tyrosine kinases

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5.1 ABSTRACT

We studied how the release of histamine and prostaglandin D_2 (PGD_2) were related in rat peritoneal mast cells, and to which extent these processes were controlled by the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, and protein tyrosine kinases. In the presence of 1 mM $CaCl_2$, the G protein-activating compound 48/80 (10 μ g/ml) evoked a transient rise in $[Ca^{2+}]_i$ and a relatively high secretion of histamine, but only a low release of PGD_2 . In contrast, 5 μ M thapsigargin (an inhibitor of endomembrane Ca^{2+} -ATPases) or 5 μ M ionomycin (a Ca^{2+} ionophore) evoked high and prolonged rises in $[Ca^{2+}]_i$, and stimulated the cells to release relatively small amounts of histamine and high amounts of PGD_2 . Stimulation of the cells with $CaCl_2$ and 10 μ M ATP^{4-} gave only minor quantities of histamine and PGD_2 , despite of the micromolar level of $[Ca^{2+}]_i$ reached. When $CaCl_2$ was replaced by EGTA, rises in $[Ca^{2+}]_i$ as well as the release of histamine and PGD_2 were reduced with each agonist, but the preference of agonists to promote either the release of histamine or PGD_2 remained unchanged. In mast cells with depleted Ca^{2+} stores, the addition of $CaCl_2$ stimulated the store-regulated Ca^{2+} entry resulting in prolonged rises in $[Ca^{2+}]_i$. Simultaneous addition of compound 48/80 and $CaCl_2$ was required for release of histamine and PGD_2 . In control cells with full Ca^{2+} stores, PGD_2 release evoked by compound 48/80 was greatly reduced by genistein and methyl-2,5-dihydroxycinnamate, two structurally unrelated inhibitors of protein tyrosine kinases, whereas histamine secretion was not influenced by these inhibitors. Similarly, with thapsigargin or ionomycin as agonist, PGD_2 release was more sensitive to the tyrosine kinase inhibitors than histamine secretion. We conclude that in activated rat peritoneal mast cells: (i) the influx of extracellular Ca^{2+} amplifies agonist-evoked rises in $[Ca^{2+}]_i$ as well as histamine secretion and PGD_2 release; (ii) the amplitude of the $[Ca^{2+}]_i$ rise does not determine the preferential effect of agonists to promote the release of histamine or PGD_2 ; (iii) the relatively high PGD_2 release evoked by thapsigargin and ionomycin is probably due to their potency to evoke a prolonged rise in $[Ca^{2+}]_i$ and to activate protein tyrosine kinases.

5.2 INTRODUCTION

Mast cells play important roles in processes like inflammation, anaphylaxis and asthma.²⁶ Activation of these cells results in the release of compounds like histamine and PGD_2 , which both induce vasodilation, increased vascular permeability and bronchoconstriction.^{11,18} Histamine and PGD_2 are released from activated mast cells by two quite different mechanisms. Histamine is stored in intracellular secretory granules and is released into the extracellular medium by exocytosis. It is generally accepted that elevation in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) as well as activation of the protein kinase C pathway are important in triggering the exocytotic event.^{13,34} The heterotrimeric G protein, G_{i3} , probably plays an additional role in this process.³ On the other hand, the principle route to produce PGD_2 , is by activation of phospholipase A_2 , a Ca^{2+} -dependent enzyme which releases arachidonic acid from membrane phospholipids.^{6,47}

Data from a previous study (*Chapter 4*) have indicated that the extent of mast cell degranulation was not related to the formation of prostanoids and hydroxy fatty acids. This is in agreement with results of others who have shown that the release of histamine and PGD₂ occur independently of each other and, hence, are regulated in different ways. For instance, it has been shown that stimulation of rat peritoneal mast cells with anti-immunoglobulin E or the Ca²⁺ ionophore A23187 resulted in a relatively high PGD₂ release and a moderate histamine secretion^{9,20,22}, whereas stimulation with bradykinin, substance P or compound 48/80 resulted in a low PGD₂ release and a high histamine secretion.^{9,22} Comparable results have been obtained in human skin mast cells.^{4,5,8} In rat peritoneal mast cells, the release of histamine and PGD₂ is known to be initiated after an increase of intracellular cytosolic Ca²⁺ concentration from basal (50–100 nM) to higher levels.^{20,25} Nevertheless, the time intervals in which these two release processes take place are often different. With various agonists, including compound 48/80, histamine secretion is detectable already at seconds after the elevation of [Ca²⁺]_i,²⁵ whereas PGD₂ generation starts much later.^{9,23} An exception appears to be the calcium ionophore A23187, which evokes a gradual and simultaneous release of both compounds that persists for several minutes.^{9,20} Taken together, this suggests that either release process requires different types of Ca²⁺ responses which, indeed, has been reported for mast cells stimulated with A23187.²⁰

We determined the relationship between histamine secretion and PGD₂ release after stimulation of rat peritoneal mast cells with different types of agonists, and questioned to what extent these processes were related to the amplitude and shape of the agonist-evoked elevation in [Ca²⁺]_i. To this end, mast cells were loaded with fura-2 and the released products after stimulation with agonists that directly or indirectly influenced intracellular Ca²⁺ levels, were measured. Cells were stimulated in the presence or absence of CaCl₂ with compound 48/80, known to activate phospholipase C in a G protein-dependent way^{27,46} and with thapsigargin, an inhibitor of the endomembrane Ca²⁺-ATPases, which in many cell types activates the so-called 'store-regulated Ca²⁺ influx pathway' independently of phospholipase C.^{36,39} Mast cells were also stimulated with agents directly permeating the plasma membrane for Ca²⁺, *i.e.*, the Ca²⁺-ionophore ionomycin, and the Mg²⁺-free form of ATP, ATP⁴⁻. The latter compound opens specific pores in the plasma membrane following binding to a specific purinergic receptor.^{33,42} Since in a variety of cell types protein tyrosine kinases are likely to be involved in the activation of phospholipase A₂¹⁹, arachidonic acid release^{21,30} and eicosanoid formation^{12,29}, we also questioned to which extent these kinases were involved in the differential release of histamine or PGD₂. Two structurally dissimilar inhibitors of protein tyrosine kinases, genistein and methyl-2,5-dihydroxycinnamate, which have been extensively tested in a number of cell types^{1,29,40,44}, were used to answer this question.

Our results indicate that the store-regulated Ca²⁺ influx pathway represents a major contribution to the Ca²⁺ signal evoked by compound 48/80 and thapsigargin, and that the influx of Ca²⁺ amplifies both histamine secretion and PGD₂ release. However, we conclude that not the amplitude of the rise in [Ca²⁺]_i but, instead, a different

involvement of protein tyrosine kinases in these two release processes primarily determines the differential release of histamine and PGD_2 .

5.3 MATERIALS AND METHODS

Chemicals

Metrizamide was purchased from Nyegaard (Oslo, Norway). Bovine serum albumin, compound 48/80 (a condensation product of *N*-methyl-*p*-methoxy-phenethylamine with formaldehyde), thapsigargin and ATP^{4-} (used in the Mg^{2+} -free form) were obtained from Sigma (St. Louis, USA). Heparin (Thromboliquine®) was from Organon Teknika (Boxtel, the Netherlands). Fura-2 acetoxymethyl ester and pluronic F-127 were from Molecular Probes (Eugene, USA) and ionomycin was from Serva (Heidelberg, FRG). All chemicals were of analytical grade and were predominantly obtained from Merck (Darmstadt, FRG). Water was purified with a milli-Q purification unit from Millipore (Bedford, USA). Methyl-2,5-dihydroxycinnamate and genistein were obtained from Sigma and were freshly dissolved into dimethyl sulfoxide before each experiment.

Mast Cells

Mast cells were isolated from retired breeder Wistar rats (Charles River Laboratories, Sulzfeld, FRG) according to the procedure described in Chapter 2. Five to ten rats were used for each experiment. Mast cells were finally resuspended in buffer pH 7.4, consisting of 136 mM NaCl, 2.7 mM KCl, 10 mM Hepes, 5 mM glucose and 0.1% (w/v) bovine serum albumin (BSA) to a final concentration of $2 \cdot 10^6$ cells/ml. Mean mast cell number obtained per rat was about $8 \cdot 10^5$, which was sufficient to carry out 2 to 3 incubations.

Measurements of Fura-2-fluorescence

Suspended mast cells were loaded with fura-2 acetoxymethyl ester (3 μM) in the presence of the dispersing agent pluronic F-127 (1 mg/ml) under slow rotation at 20 °C for 45 min, as described earlier for rat platelets.¹⁶ In order to optimize the fura-2-loading procedure, the incubation was carried out in buffer pH 7.4 supplemented with 5% (w/v) BSA. After centrifugation at $195 \times g$ for 5 min, the fura-2-loaded cells were resuspended in buffer pH 7.4 and brought into a cuvette to a final concentration of 10^5 cells/ml. Changes in fura-2-fluorescence were measured at an emission wavelength of 500 nm, while the excitation wavelength was switched continuously between 340 and 380 nm. Fluorescence data were collected every 2 s and processed by a personal computer. Occasionally, divalent cation influx was measured by following the quenching of fura-2-fluorescence that occurred when externally added Mn^{2+} entered the cells.¹⁵ In this case, the excitation wavelength was fixed at 360 nm. Calibration values for the calculation of $[\text{Ca}^{2+}]_i$ were collected after the addition of 0.1% (w/v) Triton X-100 or digitonin in the presence of 1 mM CaCl_2 , followed by 10 mM EGTA and 50 mM Tris/HCl (pH 8.3), according to the procedure of Grynkiewicz *et al.*¹⁴ In all experiments, we checked for possible compartmentalization of the loaded fura-2:

ATP⁴⁻ (10 μ M) in the presence of 1 mM CaCl₂ always evoked an immediate and high Ca²⁺ response, indicating that most of the dye was located in the cytosol.

Mast Cell Activation

Fura-2-loaded cells (2.5·10⁵ cells/2.5 ml) were activated in plastic cuvettes at 37 °C at a stirring rate of 150 rpm. Agonists were added from 1000-fold stock solutions. Calcium measurements were performed continuously or at time intervals indicated below. Samples of 500 μ l were withdrawn from the incubation mixture, immediately layered onto 200 μ l of silicon oil (d=1.049) and centrifuged in an Eppendorf centrifuge at 15,000 $\times$ g for 10 s. The supernatants were collected for the determination of histamine or PGD₂, and were stored at -70 °C until analysis. Samples for analysis of PGD₂ were methoximated directly after the experiment, according to the instructions of the kit. Total releasable histamine was measured by solubilization of a subsample of the cells with 0.1% (w/v) Triton X-100 and subsequent centrifugation of residual cell material at 15,000 $\times$ g for 10 s. The degrees of histamine secretion and PGD₂ release were corrected for the amounts of histamine and PGD₂ present in the supernatant of unstimulated cells, which values were constant within each experiment and attributed to <7% of total releasable histamine and <2 ng PGD₂/10⁶ cells, respectively.

Store-depleted Mast Cells

For a subset of experiments, mast cells with depleted Ca²⁺ stores were prepared. To this end, the cells were loaded with fura-2 as described above, except that ionomycin (5 μ M), thapsigargin (100 nM) and EGTA (0.2 mM) were added 15 min after the addition of the fura-2 acetoxymethyl ester. These additions did not interfere with the uptake or deesterification of the fura-2 ester (*data not shown*) and resulted in a depletion of the intracellular Ca²⁺ store content of 85 $\pm$ 5% and in a reduction of the releasable histamine content of 15 $\pm$ 5% (mean $\pm$ SD, *n*=4). After 30 min of incubation, the cells were centrifuged at 195 $\times$ g for 5 min, washed twice with buffer pH 7.4 and resuspended at a concentration of 10⁶ cells/ml. The content of the internal Ca²⁺ stores was estimated from the maximal increase in [Ca²⁺]_i evoked by ionomycin (5 μ M) in the presence of EGTA (1 mM). Because of the low dose of thapsigargin used during the store depletion procedure, mast cells still responded well to higher doses and to compound 48/80.

Determination of PGD₂ and Histamine

PGD₂ was measured with an enzyme-linked immunoassay kit purchased from Cayman Chemical (Ann Arbor, USA). Histamine was determined by HPLC as described in Chapter 2. The lower detection limits were 0.3% of total releasable histamine and 1 ng PGD₂/10⁶ cells.

Statistical Analysis

To evaluate the effects of inhibitors, data were compared to those of control incubations, using an Analysis of Variance. *P* values $\leq$ 0.05 were considered to be statistically significant. Data are presented as mean values $\pm$ SEM.

5.4 RESULTS

Calcium Responses of Rat Peritoneal Mast Cells

Fura-2-loaded mast cells were stimulated with non-lytic doses of various agonists in the presence of EGTA or CaCl_2 , and changes in $[\text{Ca}^{2+}]_i$ were measured by spectrofluorometry. Calcium responses in the presence of 1 mM EGTA were considered to reflect the release of Ca^{2+} from internal Ca^{2+} stores into the cytosol. Responses in the presence of 1 mM CaCl_2 were considered to reflect internal Ca^{2+} mobilization in combination with influx of Ca^{2+} from the extracellular medium.

In the presence of EGTA, compound 48/80 (10 $\mu\text{g}/\text{ml}$) gave a rapid and transient increase in $[\text{Ca}^{2+}]_i$ (Fig. 5.1A), probably caused by $\text{Ins}(1,4,5)\text{P}_3$ -evoked depletion of the intracellular Ca^{2+} stores.^{27,46} Stimulation with thapsigargin (5 μM) resulted in a slow and prolonged rise in $[\text{Ca}^{2+}]_i$ (Fig. 5.1B), pointing to gradual depletion of the Ca^{2+} stores by inhibition of the thapsigargin-sensitive Ca^{2+} -ATPases in the store membranes. Incubation with ionomycin (5 μM) resulted in an increase in $[\text{Ca}^{2+}]_i$ which was transient in 4 out of 6 experiments (Fig. 5.1C) and was not reversed in the other two experiments. The Mg^{2+} -free ATP^{4-} (10 μM) gave no significant Ca^{2+} response in the presence of EGTA (Fig. 5.1D), indicating that ATP^{4-} at this concentration did not evoke store depletion.

In the presence of CaCl_2 , stimulation of the cells with compound 48/80 resulted in a transient increase in $[\text{Ca}^{2+}]_i$ with a similar shape but an amplitude which was almost ten-fold higher (Fig. 5.2A) when compared to stimulation in the absence of extracellular Ca^{2+} (Fig. 5.1A). Similarly, thapsigargin induced comparably shaped Ca^{2+} signals in the presence (Fig. 5.2B) and absence (Fig. 5.1B) of CaCl_2 , although the level of $[\text{Ca}^{2+}]_i$ reached was much higher when CaCl_2 was present. Stimulation of the cells with ionomycin and CaCl_2 resulted in a micromolar level of $[\text{Ca}^{2+}]_i$ which remained high for at least 3 min. (Fig. 5.2C). ATP^{4-} in combination with CaCl_2 gave an immediate and high increase in $[\text{Ca}^{2+}]_i$ (Fig. 5.2D), indicating that this agonist promoted the influx of Ca^{2+} , despite its inability to evoke store depletion (Fig. 5.1D).

So far, the results suggest that depletion of the intracellular Ca^{2+} stores by compound 48/80 and thapsigargin was accompanied by a considerable influx of Ca^{2+} from the extracellular medium. Additional support that these agonists opened divalent cation channels in the plasma membrane came from experiments in which we added Mn^{2+} to the incubations. In many cells, including mast cells, Mn^{2+} enters the cytosol through the same pathways as Ca^{2+} , the appearance of which results in rapid quenching of fura-2-fluorescence.¹⁰ In these conditions, subsequent stimulation of the cells with compound 48/80 and thapsigargin, resulted in two distinguishable decreases in fura-2-fluorescence, indicating that both compounds caused Ca^{2+} influx (*data not shown*). Furthermore, the Ca^{2+} entry blocker NiCl_2 (5 mM) appeared to inhibit the CaCl_2 -dependent part of the Ca^{2+} responses evoked by both compound 48/80 and thapsigargin (*data not shown*).

Table 5.1: Effects of Various Agonists on $[Ca^{2+}]_i$, Histamine Secretion and PGD_2 Release

Agonist	Addition	Increase in $[Ca^{2+}]_i$ (nM)		Histamine secretion (% of total)		PGD_2 release (ng/ 10^6 cells)	
		2 min	10 min	2 min	10 min	2 min	10 min
Compound 48/80	CaCl ₂	496 ± 57	275 ± 37	68 ± 5	72 ± 5	4 ± 1	7 ± 1
	EGTA	30 ± 2	5 ± 3	17 ± 4	20 ± 4	<1	<1
Thapsigargin	CaCl ₂	460 ± 140	1156 ± 314	15 ± 2	18 ± 2	13 ± 2	15 ± 2
	EGTA	71 ± 20	90 ± 17	5 ± 1	15 ± 2	9 ± 2	10 ± 3
Iono:mycin	CaCl ₂	691 ± 215	869 ± 579	9 ± 3	13 ± 3	22 ± 3	30 ± 2
	EGTA	33 ± 11	29 ± 7	<0.3	<0.3	<1	<1
ATP ⁴⁻	CaCl ₂	896 ± 117	1420 ± 210	5 ± 1	4 ± 2	<1	<1
	EGTA	11 ± 11	12 ± 12	<0.3	<0.3	<1	<1

Mast cells were stimulated with compound 48/80 (10 μ g/ml), thapsigargin (5 μ M), ionomycin (5 μ M) or ATP⁴⁻ (10 μ M) in the presence of 1 mM CaCl₂ or 1 mM EGTA, as indicated. Agonist-evoked increase in $[Ca^{2+}]_i$, secretion of histamine (% of total releasable histamine) and release of PGD_2 (ng released/ 10^6 cells) were determined at 2 and 10 min after the onset of activation. Detection limits were 0.3% for histamine secretion and 1 ng/ 10^6 cells for PGD_2 release. Data are mean values $\pm$ SEM (n=4).

Calcium Responses, Histamine Secretion and PGD₂ Release

Using fura-2-loaded mast cells, the rises in $[Ca^{2+}]_i$ were compared to the potency of the agonists to release histamine and PGD₂ under conditions where Ca^{2+} influx was present or absent (*i.e.*, in the presence or absence of $CaCl_2$ or EGTA, respectively). To this end, the cells were incubated at 37 °C and, at 2 and 10 min after the addition of agonists, $[Ca^{2+}]_i$ was measured after which samples were taken from the incubation mixture for the determination of histamine and PGD₂. The results are shown in Table 5.1. When extracellular Ca^{2+} was chelated with EGTA, stimulation with compound 48/80 (10 µg/ml) resulted in a relatively high histamine secretion (20% of total releasable histamine after 10 min of activation), but in a low PGD₂ release (below the detection limit of 1 ng/10⁶ cells). Stimulation with thapsigargin (5 µM) and EGTA resulted in a somewhat lower histamine secretion (15% of total histamine), whereas the PGD₂ release was relatively high (10 ng/10⁶ cells). In the presence of $CaCl_2$, the release of both histamine and PGD₂ appeared to be potentiated, when compared to the EGTA-containing incubations. Compound 48/80 with $CaCl_2$, again, evoked a relatively high histamine secretion (72% of total mast cell histamine) and low PGD₂ release (7 ng/10⁶ cells). Thapsigargin with $CaCl_2$, again, was less efficient in evoking the release of histamine (18% of total) than of PGD₂ (15 ng/10⁶ cells). Apparently, with either agonist histamine secretion and eicosanoid formation were potentiated by the higher levels of $[Ca^{2+}]_i$ reached by the entry of extracellular Ca^{2+} . This conclusion was supported by the observation that the addition of $NiCl_2$ significantly reduced the release of mast cell products induced by compound 48/80 and $CaCl_2$ (*data not shown*).

In the presence of EGTA, ionomycin (5 µM) and ATP⁴⁻ (10 µM) were unable to evoke significant formation of histamine or PGD₂. With $CaCl_2$, however, ionomycin did promote the release of relatively low amounts of histamine (13% of total releasable histamine) and high amounts of PGD₂ (30 ng/10⁶ cells). In contrast, ATP⁴⁻ gave only a marginal secretion of histamine (4% of total) and no PGD₂ release, despite the high, micromolar levels of $[Ca^{2+}]_i$ reached in this condition.

Taken together, the results indicate that the G protein-activating compound 48/80 was more efficient in evoking histamine secretion, whereas the Ca^{2+} mobilizers thapsigargin and ionomycin were more efficient in promoting the release of PGD₂. ATP⁴⁻ (10 µM) was a rather ineffective inducer for either process. Since the rises in $[Ca^{2+}]_i$ obtained with thapsigargin and ionomycin were more prolonged than the rises with compound 48/80 (Table 5.1 and Fig. 5.2), it is tempting to suggest that the relatively high PGD₂ release seen with the Ca^{2+} mobilizers is related to the prolonged high levels of $[Ca^{2+}]_i$ (*also see below*). On the other hand, the results do *not* suggest that the amplitude or the shape of the $[Ca^{2+}]_i$ signal determines the differential release of histamine or PGD₂ since, in general, the preference of agonists to promote histamine or PGD₂ release was the same in the presence or absence of extracellular $CaCl_2$.

Ca^{2+} Influx, Histamine Secretion and PGD₂ Release by Store-depleted Mast Cells

To further investigate the significance of Ca^{2+} influx for histamine secretion and PGD₂ release, fura-2-loaded mast cells with depleted Ca^{2+} stores were prepared. Stimulation of the store-depleted cells with ionomycin in the presence of EGTA

Figure 5.1: Calcium responses of mast cells in the presence of EGTA

Fura-2-loaded mast cells were stimulated with (A) compound 48/80 (10 μ g/ml), (B) thapsigargin (5 μ M), (C) ionomycin (5 μ M) or (D) ATP⁴⁻ (10 μ M) in the presence of 1 mM EGTA. Tracings are shown of changes in $[Ca^{2+}]_i$, representative for 6 experiments. Arrows indicate the addition of agonists.

Figure 5.2: Calcium responses of mast cells in the presence of $CaCl_2$

Fura-2-loaded mast cells were stimulated with (A) compound 48/80 (10 μ g/ml), (B) thapsigargin (5 μ M), (C) ionomycin (5 μ M) or (D) ATP⁴⁻ (10 μ M) in the presence of 1 mM $CaCl_2$. Tracings are shown of changes in $[Ca^{2+}]_i$, representative for 6 experiments. Note the different scale of the Y-axis when compared to Figure 5.1.

resulted in a maximal increase in $[Ca^{2+}]_i$ which was only $15 \pm 5\%$ (mean values $\pm$ SEM, $n=3$) of the increase seen in untreated control cells, indicating that most of the intracellular Ca^{2+} was removed by the depletion procedure. Determination of the histamine content in the store-depleted cells learned that no more than $15 \pm 5\%$ of the total releasable histamine was lost by the procedure.

When 1 mM $CaCl_2$ was added to store-depleted mast cells, an immediate and prolonged increase in $[Ca^{2+}]_i$ of about 300 nM was observed (Table 5.2), most probably due to the influx of extracellular Ca^{2+} through the store-regulated entry pathway. In control cells (i.e., cells with full Ca^{2+} stores), the addition of $CaCl_2$ resulted in a $[Ca^{2+}]_i$ rise of only 10 nM. Despite the high Ca^{2+} response in the store-depleted cells, $CaCl_2$ addition resulted in only a marginal release of histamine and PGD_2 . Apparently, the influx of Ca^{2+} and the resulting high levels of $[Ca^{2+}]_i$ as such were insufficient to promote a substantial release of these compounds. When compound 48/80 was added to the store-depleted cells, no Ca^{2+} signal was detected and neither histamine nor PGD_2 were released (Table 5.2). However, when compound 48/80 was added with $CaCl_2$, a sustained increase in $[Ca^{2+}]_i$ was accompanied by a high histamine secretion (56% of total releasable histamine) and high PGD_2 release (44 ng/ 10^6 cells).

Table 5.2: *Rises in $[Ca^{2+}]_i$, Histamine Secretion and PGD_2 Release in Store-depleted Mast Cells*

Addition	Increase in $[Ca^{2+}]_i$ (nM)		Histamine secretion (% of total)		PGD_2 release (ng/ 10^6 cells)	
	2 min	10 min	2 min	10 min	2 min	10 min
$CaCl_2$	325 ± 94	309 ± 123	2 ± 1	2 ± 1	2 ± 1	3 ± 1
48/80	$24 \pm 5^*$	$38 \pm 4^*$	<0.3	<0.3	<1	<1
$CaCl_2$ +48/80	613 ± 68	392 ± 53	59 ± 3	56 ± 4	36 ± 13	42 ± 16

Fura-2-loaded mast cells with depleted Ca^{2+} stores were prepared as described in Materials and Methods. The responses evoked by the addition of $CaCl_2$ (1 mM) and/or compound 48/80 (48/80, 10 μ g/ml) were determined at 2 and 10 min after activation. Data are presented as described in Table 5.1. Results are mean values $\pm$ SEM ($n=3$).

* Control incubations without compound 48/80 showed a comparable rise in $[Ca^{2+}]_i$.

Effects of Protein Tyrosine Kinase Inhibitors on the Release of Histamine and PGD_2 by Mast Cells

The role of protein tyrosine kinases in the release of histamine and PGD_2 from mast cells was studied by using two structurally unrelated inhibitors of these signaling components: methyl-2,5-dihydroxycinnamate and genistein. After pre-incubation with these inhibitors, mast cells were stimulated with compound 48/80 or thapsigargin in the presence of $CaCl_2$. Histamine secretion induced by compound 48/80 was not influenced by either inhibitor (Table 5.3), whereas the PGD_2 release by this agonist was considerably reduced. With thapsigargin as agonist, histamine secretion was moderately influenced by both methyl-2,5-dihydroxycinnamate and genistein, in contrast to the PGD_2 release which, again, was potently inhibited (Table 5.3). Similar effects of these tyrosine kinase inhibitors were found, when the cells were stimulated with ionomycin instead of thapsigargin (*data not shown*).

Table 5.3: Effects of Protein Tyrosine Kinase Inhibitors on Histamine Secretion and PGD₂ Release by Stimulated Mast Cells

Addition	Histamine Secretion (% of total)		PGD ₂ Release (ng/10 ⁶ cells)	
	Compound 48/80	Thapsigargin	Compound 48/80	Thapsigargin
DMSO	70.3 ± 2.4	23.3 ± 1.3 ^a	6.0 ± 0.7 ^a	17.3 ± 4.3 ^a
DHC	61.0 ± 5.5	14.5 ± 1.7 ^b	1.3 ± 0.3 ^b	6.0 ± 1.4 ^b
GS	66.5 ± 4.5	12.5 ± 1.3 ^b	1.0 ± 0.6 ^b	1.0 ± 0.4 ^b

Mast cells were pre-incubated with 0.2% (w/v) dimethyl sulfoxide (DMSO, vehicle control), with 5 μ M methyl-2,5-dihydroxycinnamate (DHC) or with 100 μ M genistein (GS) during 45 min. The cells were subsequently stimulated with 10 μ g/ml compound 48/80 or with 5 μ M thapsigargin in the presence of 1 mM CaCl₂. The release of histamine and PGD₂ from the cells was determined at 10 min after activation. Data are presented as described in Table 5.1 and represent mean values $\pm$ SEM (n=4). Within each column, rows with different superscripts differ significantly from each other ($P \leq 0.05$).

5.5 DISCUSSION

Role of Store-regulated Ca²⁺ Influx in Histamine and PGD₂ Release

Activation of rat peritoneal mast cells with the G protein-activating compound 48/80 and the endomembrane Ca²⁺-ATPase inhibitor thapsigargin resulted in Ca²⁺ signals, which consisted of a relatively small component representing mobilization of Ca²⁺ from internal stores and a larger component representing influx of Ca²⁺ from the extracellular medium. Several lines of evidence suggested that the Ca²⁺-influx caused by these agonists amplified the rises in [Ca²⁺]_i evoked by store depletion and occurred through the store-regulated influx pathway: (i) compound 48/80 as well as the endomembrane-active thapsigargin induced rises in [Ca²⁺]_i which were of similar shape in the presence and absence of CaCl₂, notwithstanding the higher amplitude reached with CaCl₂; (ii) upon stimulation of the cells with compound 48/80 or thapsigargin in the presence of Mn²⁺, two separate decreases in fura-2-fluorescence were observed; (iii) when extracellular Ca²⁺ was present, only the CaCl₂-dependent part of [Ca²⁺]_i rises was suppressed by the Ca²⁺ entry blocker Ni²⁺; (iv) in mast cells with depleted Ca²⁺ stores, addition of CaCl₂ was sufficient to evoke an immediate and high increase in [Ca²⁺]_i, as predicted by the store-regulated pathway.³⁵ In many other cells types in which this influx pathway is identified, including the rat mast cell line RBL-2H3^{2,7,17}, similarly, store-regulated influx is known to be initiated by G protein-mediated activation of phospholipase C and by inhibition of thapsigargin-sensitive Ca²⁺-ATPases in the store membranes. The mechanism responsible for this type of Ca²⁺ influx is still unclear, although some proposals have been made, such as the release of an intermediary messenger other than Ca²⁺ from the store compartments.^{35,37}

Our data indicate that the relatively high levels of [Ca²⁺]_i caused by entry of external Ca²⁺ potentiated the secretion of histamine as well as that of PGD₂ from mast cells. On the other hand, there were several activation conditions, where a considerable increase in [Ca²⁺]_i was not accompanied by an appreciable release of histamine or PGD₂. For instance, stimulation of mast cells with ATP⁴⁻ and CaCl₂ resulted in only marginal histamine secretion and no detectable PGD₂ release despite a micromolar rise in

$[Ca^{2+}]_i$. Furthermore, the addition of $CaCl_2$ to store-depleted cells resulted in a prolonged increase in $[Ca^{2+}]_i$ of about 300 nM, but not in the release of histamine or PGD_2 . Typically, in both cases, the Ca^{2+} responses of mast cells consisted entirely of Ca^{2+} influx, whereas under conditions where significant amounts of histamine and PGD_2 were released, *i.e.*, in mast cells activated with compound 48/80, thapsigargin or ionomycin, Ca^{2+} influx was always accompanied by release of Ca^{2+} from the intracellular stores. Thus, these results suggest that (intracellular signaling-events associated with) the influx of extracellular Ca^{2+} amplifies the release of histamine and PGD_2 , but that the influx of Ca^{2+} as such is insufficient to trigger these events. Whereas the additional factors required for exocytosis most likely include activation of protein kinase $C^{13,34}$, the signaling factors required for eicosanoid formation are less clear (see below).

Differential Release of Histamine and PGD_2 by Activated Mast cells

Mast cell-activating agonists may be divided into two types: agonists which are more efficient in evoking histamine secretion and those which are more efficient in evoking PGD_2 release. We found that compound 48/80, which directly activates G proteins, strongly promoted histamine release, whereas thapsigargin and ionomycin were more potent in promoting PGD_2 release. These preferential actions appeared to be independent of the presence or absence of external $CaCl_2$ and, thus, of the agonist-induced rise in $[Ca^{2+}]_i$. Since compound 48/80 evoked a Ca^{2+} signal that was shorter than that evoked by thapsigargin and ionomycin, these findings suggest that especially the duration of relatively high levels of $[Ca^{2+}]_i$, but not the absolute rise in $[Ca^{2+}]_i$ is a decisive factor in promoting PGD_2 release. These results are consistent with earlier observations showing that in mast cells, G protein-activating compounds like substance P, compound 48/80 and bradykinin evoke a relatively high histamine secretion^{9,22}, whereas the Ca^{2+} ionophore A23187 and anti-immunoglobulin E are more efficient in evoking PGD_2 release.^{5,9,20,22}

When $CaCl_2$ was added to store-depleted mast cells, this resulted in the release of only minor amounts of histamine and PGD_2 , despite the persistently high level of $[Ca^{2+}]_i$ reached. However, when $CaCl_2$ was added in combination with compound 48/80, the prolonged Ca^{2+} signal was accompanied by a high release of histamine and PGD_2 . This partially contrasts with the low formation of PGD_2 and high secretion of histamine, when 'control' mast cells (*i.e.*, mast cells with full Ca^{2+} stores) were activated with compound 48/80 in the presence of $CaCl_2$. The high histamine secretion with this agonist is probably caused by activation of protein kinase $C^{13,34}$ and/or the newly identified G protein, G_{i3} . It is tempting to suggest, that the high PGD_2 release is a consequence of emptying of the intracellular Ca^{2+} stores in combination with prolonged high levels of $[Ca^{2+}]_i$: compound 48/80 allowed the store-depleted cells to empty the Ca^{2+} stores, once these had been filled by the store-regulated influx pathway evoked by the addition of $CaCl_2$. Similar activation conditions were present, when cells with full stores were stimulated with thapsigargin or ionomycin in the presence of $CaCl_2$.

Roles of Protein Tyrosine Kinases in the Release of Histamine and PGD₂

We found that genistein and methyl-2,5-dihydroxycinnamate, two well-known inhibitors of protein tyrosine kinases, suppressed PGD₂ formation much more than histamine secretion in mast cells that were activated with compound 48/80 and thapsigargin. Although we did not measure protein phosphorylation patterns directly, these results suggest that tyrosine kinases are more prominently involved in the reactions leading to activation of phospholipase A₂ than in those mediating exocytosis. There is recent evidence that protein tyrosine kinases are, indeed, involved in eicosanoid formation. For instance, from experiments with the same or similar tyrosine kinase inhibitors, it has been shown that tyrosine phosphorylation events contributed to the production of prostaglandin E₂ by murine macrophages¹² and to the mobilization of arachidonic acid in platelets³⁰ and in mast cells that were activated with stem cell factor.²¹

Typically, genistein and methyl-2,5-dihydroxycinnamate strongly influenced the relative amounts of histamine and PGD₂ that were released by mast cells in response to thapsigargin: in the presence of the tyrosine kinase inhibitors, thapsigargin changed from a good PGD₂ releaser to a relatively better histamine releaser. In several other cell systems, including rat mastocytoma RBL-2H3 cells, it has been demonstrated that compounds like thapsigargin, ionomycin and A23187 cause tyrosine phosphorylation of a whole series of proteins.^{29,38,40,45,48} Thus, it is possible that the property of these Ca²⁺-mobilizing compounds to preferentially evoke PGD₂ release is related to their effect to stimulate protein tyrosine kinases.

Studies with RBL-2H3 cells have shown that the secretion of histamine evoked by A23187, thapsigargin and anti-immunoglobulin E is modulated by tyrosine kinase or phosphatase inhibitors, suggesting that these enzymes also have a regulatory function in ionophore- and antigen-evoked exocytosis.^{38,48} In agreement with this, we found that in rat mast cells histamine secretion evoked by thapsigargin and ionomycin was moderately reduced by protein tyrosine kinase inhibition, in contrast to the secretion evoked by compound 48/80, which was not influenced. The latter effect is well compatible with the evidence that signaling pathways involving protein tyrosine phosphorylation in RBL-2H3 cells act largely independently of the activation by protein kinase C.⁴¹

The cytoplasmic, 85-kDa phospholipase A₂ is considered to mediate agonist-stimulated release of arachidonic acid in many cell types.^{24,28} Recent data indicate that the activation of p85 phospholipase A₂ requires, besides elevation in [Ca²⁺]_i, a phosphorylation step which is putatively catalyzed by mitogen-associated protein kinases (MAP kinases).^{24,31} MAP kinases belong to a family of serine/threonine kinases which can be activated by phosphorylation of their tyrosine and threonine residues and may serve as convergence points in intracellular signal transduction.³² It is known that arachidonic acid mobilizing agents activate MAP kinases and cause increased phosphorylation of p85 phospholipase A₂.^{24,30} For instance, in rat RBL-2H3 cells activated with A23187 or thapsigargin³⁸ and in cultured mouse mast cells activated with anti-immunoglobulin E or stem cell factor⁴³, MAP kinases or related enzymes appeared to be phosphorylated on tyrosine. Taken together, these findings lead to the

conclusion that the potency of such agonists to favour the release of PGD₂ in mast cells might be a consequence of their ability to phosphorylate and activate MAP kinases and/or phospholipase A₂.

In summary, our results indicate that in rat peritoneal mast cells, the store-regulated influx of Ca²⁺ forms a major contribution to the agonist-evoked increase in [Ca²⁺]_i and is a potentiating factor in the release of histamine as well as PGD₂. However, the influx of Ca²⁺ as such appears to be insufficient to promote a substantial release of these compounds. Three factors may contribute to a high release of PGD₂ by mast cell agonists: (i) prolonged high levels of [Ca²⁺]_i, (ii) emptying of the intracellular Ca²⁺ stores and (iii) the phosphorylation at tyrosine of proteins involved in the phospholipase A₂-mediated signal transduction cascade. It has been reported that in human platelets, activated with Ca²⁺ ionophore or thapsigargin, tyrosine phosphorylation and dephosphorylation are controlled by the cytosolic and stored Ca²⁺ concentrations.⁴⁵ Hence, also in mast cells these three factors may not be mutually independent.

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CHAPTER 6

The Effect of Antigen-induced Mast Cell Degranulation on LDH Release in the Ischemic and Reperfused Rat Heart

CHAPTER 6

The Effect of Antigen-induced Mast Cell
Degranulation on EDH Release in the
Ischemic and Reperfused Rat Heart

6.1 ABSTRACT

In the present study, the role of mast cells in ischemia/reperfusion-induced injury to cardiomyocytes was evaluated in the isolated rat heart. Hearts were isolated from sensitized and non-sensitized rats and perfused according to Langendorff. After 30 min of normoxic perfusion, hearts were challenged with antigen, a procedure which is known to result in a massive mast cell degranulation in sensitized hearts. After another 20 min, both 'mast cell-depleted' and control hearts were subjected to 30 min of ischemia followed by 30 min of reperfusion. The release of lactate dehydrogenase (LDH) was determined to quantitate the extent of (irreversible) injury to cardiomyocytes. Coronary flow (CF) and left ventricular developed pressure (LVDP) were monitored to study the consequences of the procedures on hemodynamic parameters. It was found that both CF and LVDP significantly increased during the first min after antigen challenge. The increase in CF and LVDP values were rapidly followed by a decrease, reaching minimal values of $59 \pm 4\%$ and $85 \pm 4\%$ of those before administration of antigen, respectively, at 2-3 min after antigen challenge. Antigen challenge did not have any significant effects on CF and LVDP in control hearts. No effects of antigen challenge on LDH release were found. During reperfusion, after 30 min of ischemia, both the increase in CF and LVDP in 'mast cell-depleted' (sensitized) were not significantly different from those in control, non-sensitized, hearts. Similarly, at the end of the reperfusion-phase, CF and LVDP values in sensitized hearts were comparable to those in control hearts. Reperfusion resulted in increased LDH release, which at no point in time was significantly different between sensitized and non-sensitized hearts. The results from the present study provide no evidence that in the isolated rat heart, mast cells are prominently involved in ischemia/reperfusion-induced injury to cardiomyocytes.

6.2 INTRODUCTION

Over the past three decades, it has become clear that in the heart prolonged ischemia initiates a sequence of events that become increasingly severe and, unless halted by reperfusion, will result in irreversible myocardial injury and cell death.³ Reperfusion, although a prerequisite for survival of the ischemic tissue, is generally associated with a sudden exacerbation of injury to the myocardium.⁴ Various mechanisms such as the generation of oxygen free radicals, calcium overload and ATP depletion have been proposed to jeopardize directly or indirectly the integrity of myocardial membranes.^{7,9,11} In recent years, much attention has been paid to mechanisms contributing to the extent of ischemia/reperfusion-induced myocardial injury.

Two studies have provided indirect evidence that mast cells are involved in myocardial injury. Observations from Jolly *et al.*⁵ have indicated that in the heart *in vivo*, stabilization of resident mast cells by lodoxamide significantly reduced the extent of ischemia/reperfusion-induced myocardial injury, measured as ultimate infarct size at 24 hours postmortem. In addition, Keller *et al.*⁶ have shown that stabilization of mast cells by lodoxamide significantly reduced the extent of (irreversible) injury to cardiomyocytes

during hypoxia/reoxygenation. The mechanisms by which mast cells cause cardiac tissue injury are largely unknown, but may be due to their release of peroxidases or proteinases.⁶

We studied the role of mast cells in ischemia/reperfusion-induced injury to cardiomyocytes in a more direct way. To this end, hearts isolated from sensitized or non-sensitized rats, were challenged with antigen (trinitro-phenyl-haptenized ovalbumin, TNP-OVA) during normoxic perfusion. This procedure has been shown to specifically result in a massive mast cell degranulation in sensitized hearts, a phenomenon which is absent in non-sensitized, control, hearts.¹³ After 20 min, 'mast cell-depleted' and control hearts were subjected to 30 min of ischemia and 30 min of reperfusion. The activity of lactate dehydrogenase (LDH) in coronary effluents was determined to quantitate the extent of (irreversible) injury to cardiomyocytes. In order to study the consequences of the procedures on hemodynamic parameters, coronary flow (CF) and left ventricular developed pressure (LVDP) were monitored.

It was found that reperfusion of the ischemic heart resulted in an increased LDH release, which at no point in time was significantly different between sensitized and non-sensitized, control, hearts. The results provide no evidence that in the isolated rat heart, mast cells are prominently involved in acute ischemia/reperfusion-induced injury to cardiomyocytes.

6.3 MATERIALS AND METHODS

Sensitization Procedure

Wistar rats (body weight 180-200 g) were sensitized by an intraperitoneal injection of a 2 ml suspension of trinitro-phenyl-haptenized ovalbumin (TNP-OVA) as described by Ufkes *et al.*¹² AlPO₄ was used as adjuvant. Control, non-sensitized animals were injected vehicle instead of TNP-OVA. After 21 days, the animals were used for the experiments. Animals had free access to food and water.

Experimental Set-up

Hearts, isolated from heparinized rats (5000 IU/kg i.p.), were immediately mounted for perfusion by the Langendorff technique. Perfusion pressure was kept constant at 80 mm Hg. The perfusion buffer (pH 7.4) was Krebs-Ringer containing (in mM): NaCl (128.0), KCl (4.7), MgCl₂ (0.6), NaH₂PO₄ (0.4), NaHCO₃ (27.0), CaCl₂ (1.3) and glucose (11.0). The Krebs-Ringer solution was kept at 37 °C and equilibrated with 95%O₂/5% CO₂ (P_{O2}>80 kPa) throughout the experiment. Left ventricular pressures were measured via a water-filled balloon (HSE, Freiburg, FRG) and recorded on a Gould recorder (type 2600 S, Gould Inc., Cleveland, USA). After an equilibration period of 30 min, diastolic ventricular pressure was adjusted to 5 mm Hg. Left ventricular developed pressure (LVDP) was calculated as the difference between left ventricular systolic and end-diastolic pressure. The hearts were electrically driven at a frequency of 5 Hz. Coronary flow was measured by timed collection of the amount of effluent dripping from the heart.

Protocol

After a 30 min equilibration period, hearts ($n=6$ per group) were administered a supramaximal bolus-injection of antigen (0.8 mg TNP-OVA in a volume of 0.3 ml), directly into the perfusion stream. Normoxic perfusion was continued for another 20 min. Thereafter, hearts were subjected to 30 min of ischemia by stopping the perfusion. One min after the onset of ischemia, pacing was stopped. During the ischemic period, the temperature of the heart was kept constant at 37 °C. Hearts were reperfused for 30 min by restoring normoxic perfusion and were paced again. Coronary effluents for the determination of coronary flow and LDH content were continuously sampled during (a) the last 5 min of the equilibration period (one 5 min period), (b) five 1 min periods and three 5 min periods after antigen challenge, and (c) ten 1 min periods and four 5 min periods after reperfusion. Bovine serum albumin (3%, w/v) was added to the coronary effluents in order to improve enzyme stability. Samples were immediately stored at -70 °C.

Determination of LDH Activity in Coronary Effluents

LDH-activity in coronary effluents was determined spectrophotometrically according to Bergmeyer *et al.*¹ using a centrifugal analyzer (Cobas Bio System). LDH-activity was determined at 25 °C, pH 7.4.

Visualization of Antigen-Induced Mast Cell Degranulation

In order to visualize the effects of antigen-induced mast cell degranulation, hearts ($n=2$, for both sensitized and non-sensitized hearts) were challenged with antigen and normoxic perfusion was continued for another 20 min. Thereafter, hearts were snap-frozen in dry ice-cooled isopentane and immediately stored at -70 °C. Sections (4-6 μ m) were cut, formalin-fixed and air dried for 60 min. Sections were incubated with 0.5% toluidine blue for 2 min. Thereafter, they were rapidly dehydrated and coverslipped with Entellan®.

Statistical Analysis

Data are presented as mean values $\pm$ SEM. Comparisons between groups were made using an Analysis of Variance. P values ≤ 0.05 were considered to be statistically significant.

6.4 RESULTS

Hemodynamic Parameters

Before antigen challenge, no significant differences were observed between coronary flow (CF) values of sensitized (7.4 ± 0.4 ml/min) and non-sensitized hearts (7.0 ± 0.4 ml/min) (Fig. 6.1A). Similar results were obtained for left ventricular developed pressure (LVDP) values of sensitized (74 ± 1 mm Hg) and non-sensitized hearts (77 ± 4 mm Hg) (Fig. 6.1B). Antigen challenge did not affect CF and LVDP values in non-sensitized (control) hearts. At 20 min after antigen challenge, CF and LVDP values of these hearts attributed to 7.5 ± 0.6 ml/min and 78 ± 3 mm Hg, respectively.

Figure 6.1: Hemodynamic Parameters: Coronary Flow and Left Ventricular Pressure. Hearts isolated from sensitized (closed circles ●) or non-sensitized animals (closed squares ■) were subjected to 20 min of normoxia, 30 min of ischemia followed by 30 min of reperfusion (See Materials and Methods). At $t=0$, hearts were challenged with antigen (TNP-OVA). * indicates statistically significant from corresponding value of control hearts.

In sensitized hearts, antigen challenge resulted in a rapid increase in CF during the first 30 s (data not shown) which rapidly decreased during the next 30 s, resulting in values which were slightly less than those observed before antigen challenge (6.9 ± 0.7 ml/min) (Fig. 6.1A). Thereafter, CF further decreased, reaching minimal values at 2 min after antigen challenge (4.3 ± 0.3 ml/min). During the next 18 min, CF steadily increased, reaching values of 6.5 ± 0.5 ml/min at 20 min after antigen challenge (corresponding to CF values of $88 \pm 6\%$ of those before antigen challenge). In sensitized hearts, administration of TNP-OVA resulted in a rapid increase in LVDP which reached maximal values of 83 ± 3 mm Hg at 1 min after antigen challenge (Fig. 6.1B). Thereafter, LVDP rapidly decreased during the next 2 min, reaching minimal values at 3 min after antigen challenge (63 ± 4 mm Hg). During the next 17 min, LVDP steadily increased, reaching values of 68 ± 2 mm Hg at 20 min after antigen challenge (corresponding to values of $93 \pm 3\%$ of those before antigen challenge).

Reperfusion resulted in a transient increase in CF reaching maximal values of 10.7 ± 0.8 ml/min and 10.3 ± 0.5 ml/min in sensitized and non-sensitized hearts, respectively, at 7 min after reperfusion. Thereafter, coronary flow steadily decreased, reaching values

of 7.0 ± 0.4 in sensitized hearts and 6.1 ± 0.4 ml/min in non-sensitized hearts at 30 min after reperfusion. No significant differences were observed between CF values of sensitized and non-sensitized hearts. LVDP was highest during the first min after reperfusion, reaching values of 128 ± 15 mm Hg in sensitized hearts and 130 ± 20 mm Hg in non-sensitized hearts. LVDP rapidly decreased thereafter, reaching values of 70 ± 4 mm Hg in sensitized hearts and 78 ± 4 mm Hg in non-sensitized hearts at 30 min after reperfusion. Again, no significant differences were observed between LVDP values of sensitized and non-sensitized hearts.

LDH release

In order to quantitate the extent of (irreversible) injury to cardiomyocytes, LDH-activity was determined in coronary effluents (Fig. 6.2).

Figure 6.2: LDH release from isolated rat hearts. Hearts isolated from sensitized (closed circles, ●) or non-sensitized rats (closed squares, ■) were subjected to 20 min of normoxia, 30 min of ischemia followed by 30 min of reperfusion. At $t=0$ hearts were challenged with antigen (TNP-OVA).

During normoxic perfusion, LDH release was very low (about 25 mU/min) and did not differ between sensitized and non-sensitized hearts. After antigen challenge, LDH release remained low in both groups. Reperfusion resulted in an increased release of LDH, reaching maximal values of 288 ± 29 mU/min in sensitized hearts and 297 ± 29

mU/min in non-sensitized hearts at 3 min after reperfusion. Thereafter, LDH release decreased, reaching values of 123 ± 14 mU/min in sensitized hearts and 124 ± 10 mU/min in non-sensitized hearts at 30 min after reperfusion. Cumulative LDH release of sensitized and non-sensitized hearts during 30 min of reperfusion attributed to 4.9 ± 0.2 U/heart and 4.8 ± 0.4 U/heart, respectively. No significant differences in LDH release during reperfusion were observed between sensitized and non-sensitized hearts.

Mast Cell Degranulation

In sections from non-sensitized, control hearts, mast cell degranulation was not observed after antigen challenge. In these hearts, mast cells showed a dense granular structure (Fig. 6.3A). In sensitized hearts, antigen-challenge resulted in a massive mast cell degranulation, as indicated by the presence of numerous granules, which were observed in the vicinity of degranulated mast cells and between cardiomyocytes. In these hearts, the dense granular structure of mast cells was lost (Fig. 6.3B).

Figure 6.3: Toluidine blue staining of sections of normoxic perfused rat hearts

Hearts isolated from non-sensitized and sensitized rats (See Materials and Methods) were perfused according to Langendorff. Both non-sensitized and sensitized hearts were challenged with antigen. After 20 min, hearts were snap-frozen in dry ice-cooled isopentane. Formalin-fixed sections ($4-6 \mu\text{m}$) were incubated with 0.5% toluidine blue for 2 min. In sections from non-sensitized hearts challenged with antigen (A), mast cells showed a dense granular structure and released granules were not observed. In sensitized hearts challenged with antigen (B), a massive mast cell degranulation was observed, as indicated by the presence of numerous mast cell granules residing in the vicinity of degranulated mast cells. In these hearts, the dense granular structure of mast cells was lost.

6.5 DISCUSSION

In the present study the role of mast cells in ischemia/reperfusion-induced injury to cardiomyocytes was evaluated. Hearts, isolated from sensitized and non-sensitized animals, were perfused according to Langendorff. During normoxic perfusion, both sensitized and non-sensitized hearts were challenged with antigen (TNP-OVA). This procedure has been shown to specifically result in mast cell degranulation in sensitized

hearts.¹³ After 20 min, the 'mast cell-depleted' hearts were subjected to 30 min of global ischemia and 30 min of reperfusion.

Vleeming *et al.*¹³ have shown that in the present experimental model, approximately 1400 ng of histamine is released during the first min after antigen challenge. This was followed by the release of about 350 ng in the next two min. Thereafter, histamine release was negligible. It has been shown that in the heart, histamine is predominantly stored in mast cells.^{2,8,10} Since the total cardiac histamine content attributes to 2500 ng (see Chapter 2), it can be calculated that in the present model, approximately 70% of the cardiac mast cells have degranulated due to antigen challenge (also see Chapter 7). This notion was confirmed when sections of sensitized hearts were incubated with toluidine blue. In sensitized hearts, antigen challenge resulted in a large proportion of degranulated mast cells, showing numerous granules residing close to and at large distances from the degranulated cells. Since antigen challenge did not result in increased LDH release during the normoxic period, these results indicate that a massive mast cell degranulation under normoxic conditions was not accompanied by injury to cardiomyocytes.

We observed that in sensitized hearts, antigen challenge resulted in a transient increase in CF and LVDP, which is in agreement with the results of Vleeming *et al.*¹³ It should be noted that, since in our experiments maximal CF values were already reached at about 30 s after antigen challenge (1 min in the study of Vleeming *et al.*¹³) and the first collection of coronary effluent was at 1 min after antigen challenge, this increase is not evident from Fig. 6.1A. In the present experimental model, the antigen-evoked increases in CF and LVDP have been shown to be absent when hearts were perfused in the presence of the H₂-receptor blocker cimetidine.¹³ In addition, histamine dose-dependently increased CF and LVDP in non-sensitized hearts.¹³ Therefore, it has been proposed by these authors that the increase in CF and LVDP after antigen challenge is due to the release of histamine by mast cells. The increases in CF and LVDP were followed by a rapid decrease, reaching minimal values at 2-3 min after antigen challenge, and a steadily increase thereafter. Vleeming *et al.*¹³ have also shown that the decrease in CF and LVDP were abolished when hearts were perfused in the presence of the 5-lipoxygenase inhibitor AA-861 or the leukotriene antagonist FPL 55712, indicating that leukotrienes might play an important role in cardiac anaphylaxis. In the present study, no effects of antigen challenge on CF and LVDP were observed in non-sensitized hearts, which points to the specificity of the sensitization procedure. During reperfusion, we found no significant differences in CF and LVDP between sensitized and non-sensitized hearts, indicating that it is unlikely that mast cells are involved in the effect of ischemia/reperfusion on these parameters after restoration of flow.

It was found that during reperfusion, LDH release from 'mast cell-depleted hearts', i.e., sensitized hearts challenged with antigen during normoxic perfusion, was equal to that of reperfused, non-sensitized, hearts. This suggests that it is unlikely that mast cells contribute to the extent of acute ischemia/reperfusion-induced injury to cardiomyocytes. This observation is of particular importance, since studies of other investigators have suggested that mast cells are involved in acute hypoxia/reoxygenation-

induced injury.⁶ These authors have shown that in the isolated rat heart, the extent of hypoxia/reoxygenation-induced injury to cardiomyocytes (measured as the release of creatine kinase) correlated closely to the release of peroxidase (PO), a proposed marker of mast cell degranulation. The reason for the discrepancy between our results and those of Keller *et al.*⁶ are unknown, but may be due to differences in the experimental model (ischemia/reperfusion vs. hypoxia/reoxygenation, the latter resulting in an extent of injury to cardiac muscle cells which is about 10 times as much as that caused by ischemia/reperfusion), or the experimental conditions used to study the effects of mast cells on loss of cardiomyocyte integrity (use of 'mast cell depleted hearts' vs. use of PO as a marker of mast cell degranulation). This will be discussed in detail in Chapter 7.

In summary, we found that during normoxic conditions, mast cell degranulation had profound effects on coronary flow and left ventricular developed pressure. However, during reperfusion no differences in these hemodynamic parameters were observed between sensitized and non-sensitized hearts. In addition, during all phases of perfusion, LDH release from 'mast cell-depleted hearts' was not significantly different from that of reperfused, non-sensitized hearts. Therefore, the results provide no direct evidence that in the isolated rat heart, mast cells are prominently involved in acute ischemia/reperfusion-induced injury to cardiomyocytes. Although the present experimental set-up differed at some points from the situation *in vivo* (absence of blood cells, no extrinsic innervation), it is tempting to speculate that mast cells are of little importance in determining the extent of acute ischemia/reperfusion-induced myocardial injury *in vivo*.

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| CHAPTER 7

Lack of Evidence for a Role of Mast Cell Degranulation in Acute Hypoxia/Reoxygenation-induced Myocardial Injury in the Isolated Rat Heart

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*Charles M.C.J. van Haaster, Wim Engels, Adriaan M. Duijvestijn, Paul J.M.R. Lemmens,
Gerard Hornstra and Ger J. Van der Vusse*

Lack of evidence for a role mast cell degranulation in acute hypoxia/reoxygenation-
induced myocardial injury in the isolated rat heart

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induced injury. These authors have shown that in the newly weaned rabbit heart, the myocardium is highly sensitive to hypoxia and that the degree of myocardial damage is directly related to the duration of hypoxia. In the present study, the authors have shown that the myocardium is highly sensitive to hypoxia and that the degree of myocardial damage is directly related to the duration of hypoxia. The authors have also shown that the myocardium is highly sensitive to hypoxia and that the degree of myocardial damage is directly related to the duration of hypoxia.

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Card of Evidence for a Role of Mast Cells in Degranulation in Aorta Hypoxia Reoxygenation-induced Atherosclerotic Lesions in the Isolated Rat Heart

7.1 ABSTRACT

In the present study, the potential role of mast cell degranulation in acute hypoxia/reoxygenation-induced injury to cardiomyocytes in the isolated rat heart was evaluated. Histamine release was determined to delineate the extent of mast cell degranulation, whereas the release of creatine kinase (CK) and lactate dehydrogenase (LDH) was assessed to quantitate the extent of irreversible injury to cardiomyocytes. The suitability of peroxidase (PO) as a marker for mast cell degranulation was also evaluated. Reoxygenation resulted in a release of histamine corresponding to $6.5 \pm 0.6\%$ of total tissue content, whereas LDH, CK and PO release amounted to $30 \pm 2\%$, $28 \pm 2\%$ and $32 \pm 3\%$ of their respective tissue contents. Identical perfusion in the presence of the mast cell stabilizer lodoxamide resulted in a reduced histamine release ($2.8 \pm 0.1\%$) of total tissue content upon reoxygenation, but the release of LDH, CK or PO was not influenced. Cumulative histamine release did not correlate to the amounts of LDH, CK or PO released. Treatment with consecutive bolus injections of the mast cell-degranulating compound 48/80 during normoxic perfusion resulted in an almost complete histamine release, whereas PO release remained below detection limit. When the compound 48/80-treated hearts were subjected to hypoxia/reoxygenation, the release of LDH, CK or PO during reoxygenation, again, remained unchanged, whereas histamine release was negligible. Determination of PO-activity of freshly isolated cardiomyocytes demonstrated that the bulk of PO in rat hearts was located in this particular cell type. We therefore conclude that in the isolated rat heart, PO release is not a specific marker of mast cell degranulation. In addition, our data provide no firm evidence that in this experimental model, mast cell degranulation contributes to a significant extent to acute hypoxia/reoxygenation-induced injury to cardiomyocytes.

7.2 INTRODUCTION

In the present study the role of mast cells in a model of hypoxia/reoxygenation-induced injury to cardiomyocytes was evaluated. Mast cells are predominantly located adjacent to capillaries, small arteries and venules⁶ and are known to represent the major repository for histamine in various tissues.¹¹ Circulating histamine is taken up by the heart and is predominantly stored in resident mast cells and in a small non-releasable histamine pool which is not localized, but may be the adrenergic nerve terminals or vascular smooth muscle cells.²³ As a consequence, the distribution of histamine in the heart closely matches the distribution of mast cells^{4,27} and, therefore, the release of this substance has been used as a marker to indicate the extent of mast cell degranulation in *in vivo* and *ex vivo* studies.^{17,24}

Observations from Keller *et al.* have implicated mast cells in acute hypoxia/reoxygenation-induced injury in the isolated rat heart.¹⁸ One of the arguments in favour of this notion was that, during reoxygenation, the release of peroxidase (PO), which was used as a marker of mast cell degranulation, correlated closely to that of creatine kinase (CK), a marker of (irreversible) injury to cardiomyocytes. Recent

observations in peritoneal mast cells²⁶, however, have shed some doubt on the specificity of PO as marker enzyme of mast cells, which makes conclusions on the crucial role of resident mast cells in reoxygenation injury in the heart as found in earlier studies, less certain.¹⁸ In addition, we have previously found that mast cells did not contribute to the extent of acute ischemia/reperfusion-induced injury to cardiomyocytes (Chapter 6). The present study aims at elucidating in more detail the potential role of resident mast cells in acute hypoxia/reoxygenation-induced injury to cardiomyocytes in the isolated rat heart. Special attention was paid to the suitability of PO as marker for mast cell degranulation by comparing the release of histamine to that of PO. The release of CK and lactate dehydrogenase (LDH) were monitored as quantitative markers of irreversible injury of cardiac muscle cells.

Since the bulk of PO in the heart was located in cardiomyocytes rather than in mast cells, we conclude that this enzyme may not be used as a marker to indicate the extent of mast cell degranulation. In addition, the results indicate that in the present model, mast cell degranulation does not contribute to a significant extent to acute hypoxia/reoxygenation-induced injury to cardiomyocytes.

7.3 MATERIALS AND METHODS

Animals

Hearts were isolated from Lewis rats (body weight, 250-280 g) obtained from the Central Animal Facility of the University of Limburg. The animals had free access to food and water.

Experimental Set-up

Hearts, isolated from heparinized rats (5000 IU/kg i.p.), were immediately mounted for perfusion by the Langendorff technique.¹⁹ Perfusion pressure was kept constant at 80 mm Hg. A thin cannula was placed in the left ventricle through the apex to register left ventricular pressure (see below). The perfusion buffer contained (in mM): NaCl (118.0), KCl (4.7), MgSO₄ (1.2), KH₂PO₄ (1.2), NaHCO₃ (25.0) and CaCl₂ (2.5). (D+)Glucose (4.0) was added when indicated. In all experiments, the final pH was 7.40 (range 7.35-7.45). Temperature was kept at 37 °C throughout the experiments.

Protocol

Hearts were subjected to similar hypoxia/reoxygenation procedures as described earlier by Keller et al.¹⁸ These procedures are known to result in a high degree of injury to cardiac muscle cells.^{9,14,15} Briefly, hearts were subjected to 35 min of normoxic perfusion, during which period the perfusion buffer (containing glucose) was gassed with a mixture of 95% O₂/5% CO₂ (PO₂ >80 kPa). Thereafter, hearts were subjected to 60 min of hypoxic perfusion, during which the perfusion buffer (without glucose) was continuously gassed with a mixture of 95% N₂/5% CO₂ (PO₂ <5 kPa). Hearts were reoxygenated for 30 min by perfusion with buffer without glucose, gassed with 95% O₂/5% CO₂. Left ventricular diastolic and systolic pressures were continuously monitored with use of the catheter inserted into the left ventricular cavity which was

connected to an external pressure transducer. Left ventricular developed pressure (LVDP) was calculated as the difference between left ventricular systolic and end-diastolic pressure. Heart rate was calculated by using pressure registrations. Coronary flow was measured by timed collection of the coronary effluents. When the effect of the mast cell stabilizer Iodoxamide tromethamine (Iodoxamide, 25 μ M, a kind gift of the Upjohn Co., Kalamazoo, USA) was studied, this compound was continuously present in the perfusion buffer throughout the experiment. Normoxic control hearts were perfused for 125 min with buffer containing glucose and gassed with 95% O₂/5% CO₂. These hearts were also perfused in the presence or absence of Iodoxamide as indicated.

In a subset of experiments, the effects of a massive mast cell degranulation on cardiomyocyte integrity during normoxia and subsequent hypoxia/reoxygenation were studied. Therefore, during normoxic perfusion, hearts were treated with three consecutive bolus injections of 50 μ g of compound 48/80 (dissolved in 0.5 ml of perfusion buffer). These hearts were either freeze-clamped at the end of normoxic perfusion ($n=4$) or were subjected to hypoxia/reoxygenation ($n=4$) and freeze-clamped thereafter (*see below*).

Collection of Tissue and Effluent Samples

At the end of the experiment, atria were rapidly removed from the ventricular tissue and hearts were immediately freeze-clamped with the use of liquid nitrogen-cooled aluminum tongs. Tissue samples were stored at -70 °C.

Coronary effluents were sampled during the last 15 min of normoxic perfusion (three periods of 5 min), during 60 min of hypoxic perfusion (initially two periods of 5 min, followed by five periods of 10 min) and during 30 min of reoxygenation (ten periods of 3 min). Bovine serum albumin (3%, w/v) was added to the coronary effluent samples in order to improve enzyme stability.

When the effect of compound 48/80 on the release of myocardial enzymes and histamine was studied, hearts were treated with three consecutive bolus injections with 5 min intervals, starting at 15 min before hypoxic perfusion. Coronary effluent samples were collected during these three phases and during one 5 min interval before the first treatment with compound 48/80. Thereafter, during hypoxia and subsequent reoxygenation, coronary effluents were collected as described above.

Biochemical Analysis of Cardiac Tissue

LDH, PO and Histamine

Parts of the frozen tissue (400-500 mg) were pulverized with a stainless steel pestle and an aluminum mortar, both cooled to the temperature of liquid nitrogen. The pulverized tissue was brought into ice-cold homogenization buffer (50 mM Tris.HCl/50 mM NaCl, pH 8.5) to a final concentration of 5% (w/v). After homogenization and sonication, a sample was withdrawn for the determination of histamine (*see below*). The remaining suspension was centrifuged at 48,000 $\times g$ for 15 min (4 °C). Supernatants were collected for the determination of PO and LDH-activity. Biochemical analysis of the remaining cell-pellet demonstrated that the procedure resulted in a complete

recovery of tissue enzyme activity in the supernatant fraction (*data not shown*). PO and LDH-activity were determined spectrophotometrically using a centrifugal analyzer (Cobas Bio System). LDH-activity was determined according to Bergmeyer *et al.*³ PO-activity was determined by a commercially available kit (Kirkegaard & Perry Laboratories, USA). Horseradish peroxidase (Boehringer Mannheim GmbH, Mannheim, FRG) was used as PO reference standard. LDH-activity was determined at 25 °C (pH 7.4) and PO-activity at 37 °C (pH 5.3). Histamine content was determined by High Performance Liquid Chromatography as described in Chapter 2.

Creatine Kinase

For the determination of CK-activity in cardiac tissue, aliquots of the frozen tissue were treated as described above, except that mercapto-ethanol (0.0002%, v/v) was added to the homogenization buffer in order to protect CK from inactivation. CK-activity (determined at 25°C, pH 7.4) was determined spectrophotometrically with a commercially available CK-NAC test kit (Merck, Darmstadt, FRG) using the Cobas Bio Autoanalyzer.

Biochemical Analysis of Coronary Effluents

The activities of LDH, CK and PO were determined in freshly obtained coronary effluent samples, as described above, on the same day of the experiment. Samples for analysis of the histamine content were stored at -70 °C.

Histochemistry

Hearts for histochemistry were subjected to 20 min of normoxic perfusion. Thereafter, they were snap-frozen in dry ice-cooled isopentane and stored at -70 °C. For histochemistry, 4-6 µm thick sections were cut and air-dried for 60 min. Sections were incubated with either 0%, 0.06% or 0.15% H₂O₂, respectively, in the presence of 0.05% diaminobenzidine (DAB) in phosphate-buffered saline (PBS), pH 5.3, which is nearly the pH optimum of mast cell and leukocyte peroxidases.¹⁶ In order to compare our results to those of Keller *et al.*¹⁸, who performed the analyses at pH 6.3, additional sections were stained with DAB in PBS, pH 6.3. Slides were routinely dehydrated and coverslipped.

Isolation of Cardiomyocytes and Determination of PO and LDH activity

Cardiomyocytes were freshly isolated from adult Lewis rats (*n*=4) according to Linssen *et al.*²⁰ and were resuspended in homogenization buffer. After homogenization and sonication, a sample was withdrawn for determination of the protein content.²¹ Thereafter, samples were centrifuged at 48,000 x *g* for 15 min (4 °C) and supernatants were collected. Histamine was absent in the supernatants, indicating that mast cells did not contaminate the cardiomyocyte suspension (*data not shown*). PO and LDH-activities were determined in the supernatants and were expressed as mU/mg cellular protein and U/mg cellular protein, respectively.

Normalization of the Data

Dry weights of cardiac tissue were determined by freeze-drying aliquots of the frozen tissue overnight. Tissue contents of LDH and CK were expressed as U/g dry weight. Tissue content of PO was expressed as mU/g dry weight and histamine content as ng/g dry weight. Whenever comparison of cardiac tissue data and coronary release data was required, we assumed that the release per heart was equivalent to the release per gram wet weight, since hearts of rats of this age weigh approximately 1 g.⁵ For conversion of coronary release per gram wet weight to release per gram dry weight, an average factor of 5 was applied (based on a percentage dry weight of approximately 20% of hearts freeze-clamped immediately after removal from the body). Coronary flow was expressed as ml/min.g dry weight, also using an average conversion factor of 5.

Statistical Analysis

Data are presented as mean values $\pm$ SEM. Comparisons between groups were made using an analysis of variance. The relation between variables was determined by linear regression analysis. Values of $P \leq 0.05$ were considered to be statistically significant.

7.4 RESULTS

Hemodynamic Parameters

After 35 min of normoxic perfusion, hearts were subjected to 60 min of hypoxic perfusion and subsequently to 30 min of reoxygenation (hypoxia/ reoxygenation), both in the presence or absence of Iodoxamide. The values of coronary flow, heart rate, left ventricular developed pressure (LVDP) at the end of the normoxic perfusion phase (35 min), hypoxic perfusion phase (95 min) and reoxygenation phase (125 min) are presented in Table 7.1.

Table 7.1: Hemodynamic Parameters

Treatment	n	Time (min)	Phase	Heart Rate (Beats/min)	LVDP (mm Hg)	Coronary Flow (ml/min.g dry w)
Control	7	35	Normoxia	244 $\pm$ 11	80 $\pm$ 3	42 $\pm$ 4
		95	Hypoxia	0	0	53 $\pm$ 5
		125	Reoxygenation	0	0	41 $\pm$ 5
Iodoxamide	6	35	Normoxia	262 $\pm$ 14	80 $\pm$ 1	40 $\pm$ 2
		95	Hypoxia	0	0	58 $\pm$ 3
		125	Reoxygenation	0	0	49 $\pm$ 3

After 35 min of normoxic perfusion, hearts were subjected to 60 min of hypoxic perfusion, followed by 30 min of reoxygenation in the presence or absence of 25 μ M Iodoxamide, as indicated. Heart Rate, Left Ventricular Developed Pressure (LVDP) and Coronary Flow at the end of the normoxic perfusion phase (35 min), hypoxic perfusion phase (95 min) and at the end of the reoxygenation phase (125 min) are presented. Data are represented as mean values $\pm$ SEM. No significant differences were observed between hearts perfused in the absence (control) or presence of Iodoxamide; n refers to the number of hearts per group.

In both groups, *i.e.*, perfusion in the presence or absence of lodoxamide, hypoxia resulted in a transient increase in the coronary flow during the first 5 min of hypoxic perfusion, which rapidly decreased thereafter, reaching a relatively constant level of about 150% of normoxic coronary flow after 20 min (*data not shown*). After 6 min of hypoxia, heart rate was zero and both diastolic and systolic pressure were equal to the perfusion pressure of 80 mm Hg. Coronary flow decreased during the first 6 min of reoxygenation and was followed by a recovery phase which tended to be higher in lodoxamide-perfused hearts. Reoxygenation did not result in improvements in heart rate or LVDP in either group.

Release of Enzymes and Histamine into Coronary Effluents: Relation to Cardiac Tissue Content

Both in the presence and absence of lodoxamide, cumulative release of LDH (12 ± 2 U/g dry weight and 12 ± 3 U/g dry weight, respectively) and CK (18 ± 3 U/g dry weight and 19 ± 4 U/g dry weight, respectively) from hearts subjected to 125 min of normoxic perfusion was very low. Total normoxic tissue contents of LDH and CK amounted to 1916 ± 71 U/g dry weight and 3172 ± 90 U/g dry weight, respectively, and were not influenced by lodoxamide treatment (1904 ± 75 U/g dry weight and 3148 ± 90 U/g dry weight, respectively). Cumulative PO and histamine release under normoxic conditions remained below detection limits (<21 mU/g dry weight and <63 ng/g dry weight, respectively). Tissue PO and histamine contents amounted to 912 ± 28 mU/g dry weight and 12984 ± 606 ng/g dry weight, respectively, and were not influenced by treatment with lodoxamide (896 ± 39 mU/g dry weight and 13030 ± 716 ng/g dry weight, respectively). Similar results were obtained when normoxic control hearts were perfused without glucose during the last 95 min of perfusion (*data not shown*).

Hypoxia resulted in limited mast cell degranulation, as indicated by an increased histamine release (259 ± 29 ng/g dry weight), which was almost absent when lodoxamide was present (25 ± 14 ng/g dry weight) (Table 7.2). In contrast, PO release remained below detection limit, both in the presence or absence of lodoxamide. Hypoxia resulted in a small loss of cardiomyocyte integrity, as indicated by an increased release of LDH (36 ± 3 U/g dry weight) and CK (51 ± 4 U/g dry weight) during this period (Table 7.2). The release of LDH or CK were not influenced by the presence of lodoxamide. Enzyme and histamine release were highest during the first five min of hypoxia and rapidly decreased thereafter (*data not shown*).

Reoxygenation resulted in an increased histamine release (845 ± 72 ng/g dry weight), which corresponds to a cumulative loss of $6.5 \pm 0.6\%$ of total tissue content during this phase. Lodoxamide significantly reduced histamine release during reoxygenation ($2.8 \pm 0.1\%$), pointing to the stabilizing effects of this compound on mast cell membranes. Irrespective of the presence of lodoxamide, reoxygenation resulted in a high degree of injury to cardiomyocytes, as indicated by the loss of $30 \pm 2\%$ and $28 \pm 2\%$ of the tissue contents of LDH and CK, respectively. Similarly, PO-loss was high, irrespective whether lodoxamide was present ($32 \pm 1\%$) or not ($32 \pm 3\%$). Reoxygenation resulted in an immediate release of enzymes and histamine, with values highest during the first three min of reoxygenation (*data not shown*).

Table 7.2: Release of Enzymes and Histamine during Normoxia, Hypoxia and Reoxygenation; Effects of Iodoxamide

Variable ¹	Iodoxamide ²	n ³	Normoxia ⁴	Hypoxia ⁵	Reoxygenation ⁶	Cumulative loss ⁷	Tissue content ⁸	% Loss during Reoxygenation
LDH	-	7	3 ± 1	36 ± 3	571 ± 43	610 ± 45	1259 ± 61	30 ± 2
	+	6	3 ± 1	41 ± 2	613 ± 25	657 ± 27	1275 ± 70	32 ± 1
CK	-	7	3 ± 1	51 ± 4	895 ± 71	950 ± 71	2336 ± 102	28 ± 2
	+	6	4 ± 1	56 ± 5	946 ± 43	1006 ± 47	2389 ± 153	30 ± 1
PO	-	7	<3	<12	293 ± 27	293 ± 27	593 ± 22	32 ± 3
	+	6	<3	<12	290 ± 12	290 ± 12	584 ± 26	32 ± 1
Histamine	-	7	<9	259 ± 29	845 ± 72	1104 ± 75	11825 ± 586	6.5 ± 0.6
	+	6	<9	25 ± 14*	371 ± 16*	395 ± 24*	12161 ± 736	2.8 ± 0.1*

After 35 min of normoxic perfusion, hearts were subjected to 60 min of hypoxic perfusion, followed by 30 min of reoxygenation

¹ LDH, CK, PO refer to lactate dehydrogenase, creatine kinase and peroxidase activity, respectively

² Iodoxamide +/- refers to absence or presence of 25 µM iodoxamide in the perfusion buffer throughout the experiment

³ n refers to the number of hearts per group

^{4,5,6} Cumulative release of enzymes or histamine in coronary effluents during Normoxia (35 min), Hypoxia (60 min) or Reoxygenation (30 min). LDH and CK release are expressed as U/g dry weight, PO release as mU/g dry weight and histamine release as ng/g dry weight

⁷ Cumulative loss of enzymes or histamine during the total perfusion period

⁸ Enzyme activity/histamine content as measured in homogenates of hearts freeze-clamped at the end of the experiment: LDH and CK are expressed as U/g dry weight, PO as mU/g dry weight and histamine as ng/g dry weight

* significantly different ($P \leq 0.05$) from corresponding group perfused in the absence of iodoxamide

Release of PO and Histamine into Coronary Effluents during Reoxygenation: Relation to irreversible Loss of Cardiomyocyte Integrity

In order to delineate a possible relation between mast cell degranulation and irreversible injury to cardiomyocytes during reoxygenation, cumulative release of histamine of each individual heart was compared to that of LDH and CK (Fig. 7.1). In addition, the cumulative release of PO of each heart was compared to that of LDH and CK. LDH release correlated closely ($r=0.95$) to the release of CK (Fig. 7.1A), indicating simultaneous loss of these enzymes from cardiomyocytes into the extracellular compartment. Similar results were obtained by correlating PO release to the extent of injury to cardiomyocytes, assessed as the release of LDH (Fig. 7.1B, $r=0.92$) or CK (Fig. 7.1C, $r=0.92$). In contrast, no correlation was observed between the release of histamine and that of LDH (Fig. 7.1D, $r=0.07$) or CK (Fig. 7.1E, $r=0.06$), indicating that mast cell degranulation is not quantitatively related to the extent of injury to cardiomyocytes. Furthermore, the observation that histamine release did not correlate to PO release during reoxygenation (Fig. 7.1F, $r=0.23$) indicates that PO is not a suitable marker to indicate the extent of mast cell degranulation.

The Effect of Compound 48/80 on Myocardial Enzyme and Histamine Release during Normoxic Perfusion

In order to gain additional insight into the suitability of PO as marker of mast cell degranulation, hearts ($n=4$) were treated with three consecutive bolus injections of the mast cell-degranulating agent compound 48/80 during the normoxic perfusion period. During the 5 min interval before the first treatment, histamine and PO release were below detection limits (<1.0 mU/g dry weight and <3.0 ng/g dry weight, respectively). The first bolus injection of compound 48/80 resulted in a massive mast cell degranulation, as indicated by the release of 9010 ± 300 ng histamine/g dry weight during the first 5 min after stimulation, which corresponds with $70 \pm 3\%$ of total cardiac histamine. In contrast, PO release remained below the detection limit during this period (<3 ng/g dry weight). The majority of the remaining cardiac histamine could be released by another bolus injection of compound 48/80 (3215 ± 115 ng histamine/g dry weight). The third bolus injection resulted in only a marginal histamine release (255 ± 10 ng/g dry weight). Also after the second and third compound 48/80 treatment, PO release remained below detection limit. Analysis of the histamine content of the hearts showed that the bulk of the cardiac histamine was released by this protocol, the non-releasable histamine pool being 390 ± 20 ng/g dry weight, i.e., about 3% of total cardiac histamine pool, whereas the PO content (890 ± 40 mU/g dry weight) was equal to that of untreated, normoxic perfused hearts (912 ± 28 mU/g dry weight).

To investigate a possible detrimental effect of mast cell degranulation on cardiomyocyte integrity during normoxic perfusion, we compared the release of histamine to that of LDH. Before treatment with compound 48/80, LDH release was low (0.90 ± 0.1 U/5 min-g dry weight). Despite a massive mast cell degranulation after the first bolus injection with compound 48/80, the release of LDH into coronary effluents was not increased (0.92 ± 0.10 U/5 min-g dry weight), indicating that under normoxic conditions mast cell degranulation does not affect the integrity of

cardiomyocytes. Treatment with two consecutive bolus injections of compound 48/80 also did not result in increased LDH release.

Figure 7.1: Relationship between the release of markers of injury to cardiomyocytes and of mast cell degranulation during reoxygenation

The cumulative release of LDH of each individual heart during reoxygenation was plotted against the cumulative release of CK (A). Similarly, cumulative PO release of each heart was plotted against the cumulative LDH or CK release (B, C). Cumulative histamine release during reoxygenation was also plotted against cumulative LDH release (D), CK release (E) or PO release (F). Cumulative enzyme or histamine release of hearts perfused in the presence or absence of the mast cell stabilizer lodoxamide (25 μ M) are indicated with closed triangles ($\blacktriangle$) or closed circles ($\bullet$), respectively.

Pretreatment with Compound 48/80 during Normoxic Perfusion: Influence on Hypoxia/Reoxygenation-induced Injury to Cardiomyocytes

Thus far, the results indicate that the bulk of cardiac mast cells has degranulated after treatment with three consecutive bolus injections of compound 48/80 and that the extent of mast cell degranulation during reoxygenation is not quantitatively related to the loss of cardiomyocyte integrity. In order to gain additional insight into the role of mast cell degranulation in hypoxia/reoxygenation-induced injury to cardiomyocytes, hearts pretreated with the mast cell-degranulating agent compound 48/80 during normoxic perfusion, were also subjected to 60 min of hypoxia and 30 min of reoxygenation.

The cumulative release of LDH, CK and PO of these hearts during reoxygenation attributed to 603 ± 25 U/g dry weight, 923 U/g dry weight and 279 ± 10 mU/g dry weight, respectively. These values were not significantly different from those observed in hearts which were not pretreated with compound 48/80 (Table 7.2). Due to pretreatment with compound 48/80 during normoxic perfusion, resulting in an acute and in a severe depletion of the endogenous histamine pool, histamine release during reoxygenation remained below detection limit (<18 ng/g dry weight). The tissue contents of LDH, CK and PO in these hearts decreased to 1265 ± 65 U/g dry weight, 2369 ± 123 U/g dry weight and 604 ng/g dry weight, respectively. These values were not significantly different from the corresponding content of reoxygenated hearts which were not pretreated with compound 48/80. Moreover, histamine content of compound 48/80 pretreated hearts subjected to hypoxia/reoxygenation amounted to 400 ± 20 ng/g dry weight. This value was not significantly different from the histamine content of hearts subjected to a similar pretreatment with compound 48/80 but which were freeze-clamped just before hypoxia (see above).

Histochemical Determination of PO in Cardiac Tissue

Since the above mentioned findings strongly suggested the presence of PO in cardiomyocytes, we histochemically determined the activity of this enzyme in sections of normoxic perfused hearts. A photograph of a representative histochemical localization of PO in cardiac tissue is shown in Fig. 7.2. In control incubations (*i.e.*, in the absence of H_2O_2), staining of cardiomyocytes and mast cells was very vague and comparable to that of the interstitial space (A). In the presence of 0.15% H_2O_2 , PO-staining of both cardiac mast cells and cardiomyocytes was significantly increased. Mast cells stained heavily for PO-activity (B), whereas cardiomyocytes stained light and diffuse (C). PO-staining of both mast cells and cardiomyocytes was significantly less marked when the lower concentration of 0.06% H_2O_2 was used (*data not shown*). In order to correlate these findings to those of our experiments (*i.e.*, determination of PO-activity in coronary effluents and heart homogenates at pH 5.3), additional sections were stained at pH 5.3. The results were similar to those observed at pH 6.3: an intense staining of mast cells and a light and diffuse staining of cardiomyocytes which were both increased at elevated H_2O_2 concentrations.

Figure 7.2: Histochemical detection of peroxidase-activity in rat heart

Sections of normoxic-perfused rat hearts (6 μ m) were incubated with 0.05% DAB in phosphate-buffered saline, pH 6.3 in the presence of 0.15% H_2O_2 (see Materials and Methods). PO-activity was detected as a brown precipitate. (A), (B) and (C) refer to interstitial space, cardiac mast cell and cardiomyocytes, respectively.

Biochemical Analysis of Isolated Cardiomyocytes

To estimate the percent contribution of cardiomyocyte PO to total cardiac PO, the activity of this enzyme was assessed quantitatively in freshly isolated cardiomyocytes. Biochemical analysis demonstrated that PO-activity of these cells was 1.19 ± 0.20 mU/mg protein (Table 7.3). It was calculated that the percent contribution of cardiomyocyte PO to total cardiac PO was about 90%. Similarly, it was calculated that the vast majority of the cardiac LDH-content is present in cardiomyocytes (about 96%), which allows for the use of this enzyme as marker of loss of cardiomyocyte integrity.

Table 7.3: Estimation of the Percent Contribution of Cardiomyocyte (CMC) Enzyme Content to Total Heart Content

Enzyme ¹	Activity in isolated cardiomyocytes (U/mg protein) (measured)	Activity per gram cardiac tissue (U/g wet weight) (calculated) ²	Activity per gram cardiac tissue (U/g wet weight) (measured) ³	Contribution of CMC to total cardiac content (%) (calculated)
LDH	2.73 ± 0.31	368 ± 42	383 ± 14	96 ± 11
PO	1.19 ± 0.20 ($\cdot 10^{-3}$)	161 ± 27 ($\cdot 10^{-3}$)	182 ± 6 ($\cdot 10^{-3}$)	90 ± 15

¹ LDH and PO refer to lactate dehydrogenase and peroxidase-activity, respectively

² Assumptions: (a) contribution of other cell types (endothelial cells, fibroblasts, mast cells etc.) can be neglected (b) 1 gram of cardiac tissue corresponds to 1 ml and (c) since cardiomyocytes occupy more than 90% of the total cellular volume of the heart¹, the protein content of cardiomyocytes (CMC) constitutes the bulk of the protein present in the heart (about 150 mg/g wet weight)

³ For the conversion of LDH or PO-content per gram dry weight (Table 7.2) to the respective contents per gram wet weight, an average wet/dry weight ratio of 5 was used

7.5 DISCUSSION

In the present study, the role of mast cell degranulation in acute hypoxia/reoxygenation-induced injury to cardiomyocytes was evaluated. This model is known to result in a relatively high degree of injury to cardiomyocytes.^{9,14,15} Special attention was paid to the suitability of PO release as a marker of mast cell degranulation, which has previously been suggested to be a specific marker for this cell type.¹⁸

Several lines of evidence suggested that in the isolated rat heart, the release of PO was not a suitable marker to quantitate the extent of mast cell degranulation: (a) the release of histamine during reoxygenation did not correlate to that of PO, (b) treatment of hearts with the mast cell-degranulating agent compound 48/80 during normoxic perfusion resulted in the release of the bulk of cardiac histamine, but the release of PO remained below detection limit, and (c) perfusion in the presence of the mast cell stabilizer Iodoxamide significantly reduced histamine release during reoxygenation, whereas PO release was not influenced. The observation that PO was released in concert with the myocardial enzymes LDH and CK suggested the presence of PO in cardiomyocytes. This notion was confirmed when sections of normoxic perfused hearts were stained with DAB. It should be realized, however, that even if PO is present in cardiomyocytes at a relatively low concentration, the contribution of cardiomyocyte PO to total heart PO would most likely be much greater than mast cell-derived PO, since the latter cells are much smaller and far less in number. For instance, if one assumes that a heart of 1 gram equals 1 ml, that mast cells have an average diameter of 10 μm and a rat heart contains about 10^6 mast cells per heart (based on morphometric data from Rakusan *et al.*²⁵ and unpublished data from our own laboratory), it can be calculated that approximately 0.05% of the total cardiac volume is occupied by mast cells. Anversa *et al.*¹ measured the relative space-distribution in the heart and found that approximately 60% of the total volume of the heart was occupied by cells, which implies that mast cells occupy approximately 0.08% of the total cellular volume of the heart. Therefore, even if one assumes that the PO-activity of mast cells is 10 times as much as that in cardiomyocytes, this activity is restricted to <1% of the total cellular volume of the heart. Since about 90% of the total cellular volume of the heart is occupied by cardiomyocytes, the PO-content of these cells, although relatively low, may account for the bulk of PO-activity present in the heart. This notion was confirmed when we estimated the percent contribution of cardiomyocyte PO to total cardiac PO, which was found to be about 90%. The presence of PO in cardiomyocytes suggests a role of this enzyme in this particular cell type. Although the specific role of PO in cardiomyocytes was not elucidated in full detail, the enzyme in these cells is most likely involved in scavenging endogenously produced H_2O_2 .

Our results did not provide evidence for a role of mast cells in *acute* hypoxia/reoxygenation-induced injury to cardiomyocytes: (a) we observed no correlation between the release of histamine and the release of LDH or CK during reoxygenation, (b) when hearts were perfused in the presence of Iodoxamide, histamine release during reoxygenation was significantly reduced, but the release of LDH or CK remained unchanged, (c) during reoxygenation, the release of LDH, CK or PO from hearts in which

the majority of mast cells had degranulated due to treatment with compound 48/80 during normoxic perfusion (i.e., 'mast cell-depleted hearts'), was identical to that of non-treated, control hearts. These results strengthen our earlier notion (Chapter 6) that, most probably, mast cells are of minor importance in mediating the extent of acute ischemia/reperfusion-induced injury to cardiomyocytes *in vivo*. However, since mast cells may potentially release a variety of proinflammatory substances, such as tumor necrosis factor α (TNF α) and various interleukins^{10,12}, these cells may be involved in longer-term effects of ischemia/reperfusion, such as the infiltration of leukocytes into the tissue. The infiltration of these cells into previously ischemic myocardial tissue is known to play an important role in the development of myocardial injury following ischemia/reperfusion.^{2,7,22} This phenomenon may be due to the fact that leukocytes (especially neutrophils) release a variety of harmful substances, such as hydrogen peroxide and proteases, which may directly injure cardiac muscle cells.⁸ The possible role of mast cells in leukocyte-adhesion process will be discussed in Chapter 8.

In the present study we have focused on the potential role of mast cell degranulation in irreversible injury to cardiomyocytes. Damage of other structures in the heart, such as the extracellular matrix, might have occurred during acute hypoxia/reoxygenation. Studies of Gruber *et al.*¹³ have suggested that products released from mast cells, such as tryptase, may affect the integrity of the extracellular matrix by virtue of its direct action on glycoproteins. Therefore, it cannot be excluded that degranulation of mast cells during acute hypoxia/reoxygenation contributes to damage of the extracellular matrix, if any, in the affected heart. However, acute damage inflicted upon cardiomyocytes seems not to be influenced by resident mast cells.

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| CHAPTER 8

Mast Cells and the Induction of Endothelial Leukocyte-Adhesion Molecules in Vitro

Charles M.C.J. van Haaster, Josien G. Derhaag, Wim Engels, Paul J.M.R. Lemmens, Annemie P. Gijzen, Gerard Hornstra, Ger J. Van der Vusse and Adriaan M. Duijvestijn

Mast cells and the induction of endothelial leukocyte-adhesion molecules in vitro

(Submitted for Publication)

8.1 ABSTRACT

We approached the possible role of cardiac mast cells in the expression of endothelial leukocyte-adhesion molecules ICAM-1, VCAM-1 and E-selectin in the intact heart with an in vitro rat experimental model. Isolated rat peritoneal mast cells and rat heart endothelial cell lines were used for ICAM-1 and VCAM-1 induction studies, whereas for E-selectin studies human umbilical vein endothelial cells (HUVEC) were used, since no suitable antibodies against rat E-selectin were available. Mast cells were incubated either in direct cell-cell contact with endothelial cell monolayers (contact studies) or separated from endothelial cells through a permeable membrane (transwell studies). To study the effects of mast cell degranulation on the induction of leukocyte-adhesion molecules, mast cells were activated with compound 48/80. Both in contact and transwell studies, mast cells induced the expression of VCAM-1 and E-selectin in endothelial cells, whereas the induction of ICAM-1 was marginal. The induction of VCAM-1 and E-selectin expression was not related to mast cell degranulation or to mast cell-derived products such as histamine, but seemed to be provoked by, so far, undefined substance(s) constitutively released by these cells. The direct contact between mast cells and endothelial cells potentiated the induction of VCAM-1 and E-selectin in endothelial cells. The results suggest that in the heart mast cells may play a distinct role in the concert of early inflammatory events leading to the expression of endothelial cell adhesion molecules involved in attracting blood leukocytes.

8.2 INTRODUCTION

Numerous reports have shown that ischemia/reperfusion triggers leukocytes to infiltrate the myocardium where they increase the extent of myocardial injury^{8,20,26,27,29}, possibly by releasing cytotoxic compounds such as proteases and reactive oxygen species, which may directly injure cardiomyocytes.⁹

The extravasation of leukocytes during an inflammatory response is a multistep-process, which requires the interaction of these cells with the vascular endothelium. This process consists of at least two distinct adhesive events. The first event involves rolling of leukocytes along the endothelial membrane, a process which is mediated by selectins (P-selectin, E-selectin and L-selectin) and their respective sialylated carbohydrate ligands.² P-selectin and E-selectin are expressed by endothelial cells, whereas L-selectin is expressed by leukocytes. E-selectin mediates the interaction between endothelial cells and leukocytes, e.g., neutrophils, monocytes and memory CD4⁺ lymphocytes.^{4,16,28} After rolling, firm adhesion is accomplished by endothelial adhesion molecules with immunoglobulin domains such as VCAM-1 and ICAM-1, which mediate adherence of leukocytes bearing β 1-integrins such as VLA-4 (CD 49d) and β 2-integrins such as LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18).^{7,21,22} VCAM-1 has been shown to mediate the adhesion of lymphocytes, monocytes, eosinophils and basophils to endothelial cells^{4,22}, whereas ICAM-1 mediates adhesion of all leukocytes.^{6,7}

The trigger of an inflammatory response, ultimately resulting in extravasation of leukocytes to a particular target tissue, requires a signal that is presumably released from the injured site. The mast cell, by virtue of its perivascular localization and its capacity to release a variety of potent inflammatory mediators such as histamine, platelet activating factor (PAF), $\text{TNF}\alpha$ and various interleukins such as IL-4 and IL-8¹¹, may serve such function. Indeed, products released from mast cells have been shown to increase the expression of E-selectin in human skin^{12,32} and to cause extravasation of leukocytes in mouse skin and the hamster cheek pouch.^{25,33} Since ischemia/reperfusion is accompanied by mast cell degranulation and hence, by the release of mast cell products^{18,19}, longer-term effects of this process *in vivo* might involve the extravasation of leukocytes and, consequently, mast cells may contribute to the extent of ischemia/reperfusion-induced injury to cardiomyocytes. In the process of leukocyte extravasation, the induction of endothelial leukocyte-adhesion molecules is an important step.

We approached the potential role of cardiac mast cells in the induction of endothelial leukocyte-adhesion molecules such as ICAM-1, VCAM-1 and E-selectin in the intact heart with an *in vitro* rat experimental model. Since isolation of mast cells from cardiac tissue usually results in low yield and high impurity^{1,23}, peritoneal mast cells were used instead. It should be noted that the latter cells, similar to cardiac mast cells, belong to the connective tissue-type. Mast cells were studied for their capacity to induce the expression of ICAM-1, VCAM-1 and E-selectin in cultured endothelial cells. For ICAM-1 and VCAM-1 expression studies, cultured endothelial cells from rat heart origin were used, whereas for E-selectin studies human umbilical vein endothelial cells (HUVEC) were used, since no suitable rat E-selectin antibodies were available. Since *in vivo* mast cells are located either in direct contact with or in the close vicinity of vascular endothelial cells, in experiments, mast cells were incubated either in direct contact with endothelial cells, or separated from these cells through a permeable membrane. Mast cells were activated with compound 48/80 in order to study the possible role of mast cell degranulation in the induction of endothelial leukocyte-adhesion molecules.

The results show that in this experimental set-up products released from mast cells induced the expression of VCAM-1 and E-selectin in endothelial cells, whereas the induction of ICAM-1 by products released from mast cells was marginal. The induction of VCAM-1 and E-selectin expression was not related to mast cell degranulation and mast cell-derived products such as histamine, but seemed to be provoked by (an) so far undefined substance(s) constitutively released from mast cells. Direct contact between mast cells and endothelial cells potentiated the expression of VCAM-1 and E-selectin.

8.3 MATERIALS AND METHODS

Endothelial Cells and Cell Culturing

Experiments were performed with various rat heart endothelial cell (RHEC) lines produced from cultures of heart endothelial cells isolated by *ex vivo* perfusion with collagenase.⁵ These lines have been characterized for their endothelial nature and expression of cell adhesion molecules after stimulation with cytokines.⁵ For the studies

described here, two RHEC lines were used: (1) RHEC-3, which expressed low levels of ICAM-1 under non-stimulatory conditions, expression levels that could strongly be enhanced by cytokine stimulation and (2) RHEC-11^{V+} (RHEC-11), a recently prepared line from RHEC-11 which shows a high expression of VCAM-1 after combined cytokine stimulation. When the effects of mast cells on ICAM-1 expression were studied, RHEC-3 was used, whereas RHEC-11 was used to investigate the effects of mast cells on VCAM-1 expression. Endothelial cell (EC) lines were cultured in a CO₂ incubator at 37 °C in culture flasks (Costar, Cambridge, UK). EC culture medium consisted of 40% RPMI 1640 (Gibco BRL, Life Technologies B.V, Breda, the Netherlands), 40% M199 (Gibco), 20% heat-inactivated foetal calf serum (FCS, Integro, Belgium), bovine brain extract (14.93 mg/ml)¹⁷, 10 mM NaHCO₃, penicillin (50 I.E./ml), streptomycin (50 µg/ml), fungizone (2 µg/ml), Hepes (10 mM), heparin (20 I.E./ml) and L-glutamin (2 mM). Endothelial cells were passaged routinely in 1 to 3 dilution. EC-viability was always >95% as assessed by Trypan-blue exclusion. HUVEC were isolated according to Vossen *et al.*³¹ and were used in passage 3 to 4.

Mast cell Isolation

Mast cells were isolated from the peritoneal cavity according to a slightly modified version of that described in Chapter 2. Briefly, rats were anaesthetized with diethyl ether and 20 ml of sterile lavage buffer (150 mM NaCl, 8 mM Na₂HPO₄·2H₂O, 1.6 mM KH₂PO₄, 0.1% gelatin (w/v) and 10 I.E./ml heparin, pH 7.4) was injected intraperitoneally using a sterile infusion syringe (Abbocath-T 14Gx51 mm, Abbott, Ireland). After gentle massaging, the lavage buffer was collected through the syringe and transferred into a sterile tube. The isolation procedures were identical to those described in Chapter 2, except that they were performed in a laminar-flow cabinet using sterile materials and solutions. At the end of the procedure, cells were resuspended in EC culture medium to a final concentration of 10⁶ cells/ml and were counted using a Coulter Counter (Coulter Electronics, Krefeld, FRG).

Mast cell/Endothelial Cell Incubations

Endothelial cells (EC) were seeded into fibronectin-coated (200 mg/ml fibronectin incubated for 30 min) tissue-culture dishes (6 well-plates, Costar) and were subsequently incubated at 37 °C in an atmosphere of 95% air/5% CO₂ in 2.5 ml EC culture medium. At confluency, the medium was replaced by 2.5 ml fresh EC culture medium.

In contact studies (*contact incubations*), mast cells were added to the culture dishes to a final concentration of 2·10⁵ cells/ml. In experiments in which mast cells were separated from endothelial cells (*transwell incubations*), inserts (membrane thickness 10 µm, pore size 0.4 µm, Costar) were used to which mast cells were added. The incubation volume in both conditions was 2.5 ml. Inserts were presoaked in EC culture medium. When indicated, mast cell degranulation was induced by addition of compound 48/80 (10 µg/ml). Endothelial cells were also incubated in the presence of cytokines (a combination of 400 U/ml TNFα and 200 U/ml IFNγ) or lipopolysaccharide (LPS, 10 µg/ml). In addition, endothelial cells were incubated with two concentrations of histamine (3.5 µM or 35 µM). The effects of mast cells on ICAM-1 and VCAM-1

expression were studied after 16 hours incubation, which was shown in preliminary experiments to be a time point falling within the peak expression of ICAM-1 and VCAM-1 in RHEC (*data not shown*). When the effects of mast cells on the expression of E-selectin were studied, incubations were carried out for 4 hours, a time point falling within the peak expression induced with cytokines. At the end of the experiment, the medium was collected and transferred into Eppendorf cups. After centrifugation at $15,000 \times g$ for 30 s, supernatants were stored at -70°C until analysis for the histamine content.

Cardiomyocytes and Heart Fibroblasts

Cardiomyocytes (CMC) were isolated from adult rat hearts according to Linssen et al.¹⁵ The cells were studied for their capacity to increase ICAM-1 and VCAM-1 expression in RHEC-3 and RHEC-11, respectively. CMC were added to confluent monolayers of endothelial cells in contact incubations (final concentrations $5 \cdot 10^4$ and $5 \cdot 10^3$ cells/incubation, $n=2$). Fibroblasts were derived from a cell-line (CFLC) prepared from fibroblast-cultures of rat heart origin.¹⁵ CFLC were also tested for their capacity to increase ICAM-1 and VCAM-1 expression in transwell incubations (final concentration $5 \cdot 10^5$ cells/incubation, $n=2$).

Determination of Histamine Content

The extent of mast cell degranulation was quantitated by determination of the percentual histamine release in mast cell/EC incubations. Histamine was determined by High Performance Liquid Chromatography as described in Chapter 2. Total releasable mast cell histamine was determined after lysing the cells with 0.1% (w/v) Triton X-100 (see Chapter 5). This amount of histamine (about $35 \mu\text{M}$) was added to EC monolayers and was used as a reference for the calculation of the percentual histamine release in mast cell/EC incubations.

Cytocentrifuge Preparations

Endothelial cell monolayers were washed with phosphate-buffered saline (PBS) and subsequently treated with $100 \mu\text{l}$ of a trypsin (0.25%)/ EDTA (0.2%) solution. After 1 min, trypsin was inactivated by addition of 5 ml of a solution consisting of M199 (90%, v/v), foetal calf serum (10%), penicillin (100 I.E./ml), streptomycin (100 $\mu\text{g/ml}$), L-glutamin (2 mM) and Hepes (25 mM). After centrifugation at $200 \times g$ for 10 min, endothelial cell suspensions were routinely fixed with 1% (w/v) paraformaldehyde in PBS for 30 min at 4°C . After three consecutive washing-steps with PBS, the cells were resuspended in 2.5 ml of EC culture medium. Preparations were made in a Cytocentrifuge (Cytospin 3, Shandon, UK) used at 680 rpm for 10 min. Preparations were stored in an exsiccator.

Immunoperoxidase Staining

Staining was performed on paraformaldehyde-fixed cytocentrifuge preparations, using a two-step immunoperoxidase technique. In preliminary experiments, saturating concentrations of the antibodies diluted in PBSA (PBS containing 0.05% bovine serum

albumin, BSA) were determined (*data not shown*). Preparations of RHEC were incubated with 1A29, a mouse monoclonal antibody (mAb) against rat ICAM-1 (donated by Dr. Miyasaka, Osaka University, Japan), 5F10, a mouse mAb against rat VCAM-1 (donated by Drs. R. Lobb and P. Chisholm, Biogene, Cambridge, USA) or irrelevant isotype-matched control antibodies. HUVEC cytocentrifuge preparations were incubated with BBIG-E4 (R&D Systems, Abingdon, UK), a mouse mAb against human E-selectin. Preliminary experiments with CL-3 (a mouse monoclonal antibody against human E-selectin, a kind gift from Dr. D.C. Anderson, Upjohn Co., Kalamazoo, USA) gave results which were comparable to those observed with BBIG-E4 (*data not shown*). 6.5B5, a monoclonal mouse anti-human VCAM-1 antibody was donated by Dr. D. Haskard (University of London, UK). Incubation with first-step antibodies was carried out for 60 min in a humidified chamber. Thereafter, preparations were washed with PBS and incubated for 30 min with peroxidase-conjugated second-step antibodies (rabbit F(ab)₂ anti-mouse IgG, Dako, Copenhagen, Denmark) in PBSA. When RHEC were stained, normal rat serum (3%, v/v) was added to prevent non-specific binding of the rabbit anti-mouse IgGs, whereas normal human serum was added when HUVEC were stained. After washing with PBS, preparations were incubated for 10 min with diaminobenzidine (DAB) in PBS (0.05%, w/v) in the presence of 0.5% H₂O₂ (v/v). The reaction was stopped by placing the preparations in water. After counterstaining with hematoxylin, preparations were routinely dehydrated and coverslipped with Entellan® (Merck, Darmstadt, FRG). Quantification of positive cells occurred microscopically by counting a minimum of 200 cells over at least two fields, or in case of low numbers of positive cells (*i.e.*, ICAM-1 expression), a minimum number of 10 positive cells.

Histochemistry on Cardiac Tissue Sections

In order to remove blood cells from the tissue, hearts were shortly (30 s) perfused according to Langendorff. Hearts were fixed by perfusion with 15 ml of formalin (50% in PBS, v/v). After overnight incubation in formalin, parts of the tissue were processed for paraffin embedding using a Histokinette.

Endothelial cells were visualized by incubation of the sections with biotinylated Griffonia Simplificolia (BS-I, Sigma, St. Louis, USA), a lectin which specifically recognizes terminal α -galactosyl residues on endothelial cells. Deparaffinized sections (4 μ m) were incubated overnight with BS-I in the presence of BSA (1%, w/v) and Tween-80 (0.1%, v/v). After washing with PBS, sections were incubated with peroxidase-labeled avidin for 30 min. After washing with PBS, sections were incubated with DAB (0.05%, w/v) in the presence of 0.15% H₂O₂ until staining was considered optimal. The reaction was stopped by placing the sections in water. Thereafter, sections were incubated with 0.05% (w/v) toluidine blue for 5 min to visualize mast cells. Sections were rapidly dehydrated and coverslipped with Entellan®.

Statistical Analysis

Results are expressed as the percentage of positive cells after correction for expression levels in control endothelial cell incubations, *i.e.*, in the absence of mast cells (mean

values $\pm$ SEM). Statistical analysis was performed using the non-parametric Mann-Whitney U-test. P values ≤ 0.05 were considered to be statistically significant.

8.4 RESULTS

Localization of Cardiac Mast cells

Sections of formalin-fixed cardiac tissue were stained with BS-I and toluidine blue to visualize endothelial cells and mast cells, respectively. It was found that mast cells were located around large- and medium-sized blood vessels in the cardiac connective tissue strands. In addition, mast cells were also found to a lower extent around capillaries and postcapillary venules in the myocardium. More specifically, mast cells were located in the close vicinity of and sometimes in contact with endothelial cells (Fig. 8.1A).

Figure 8.1 (A): Paraffin-sections of rat heart stained with BS-I lectin for visualization of endothelial cells and toluidine blue for mast cells. Arrows indicate mast cells in contact with or in the close vicinity of a blood vessel (v) (B,C,D): Cyto-centrifuge preparations of EC cultures: immunoperoxidase staining with hematoxylin counterstaining. Large arrows indicate strongly positive cells, small arrows moderately positive cells. Asterisks indicate negative cells. (B): VCAM-1 induction in RHEC-11 by cytokines, showing positivity in the majority of the cells (C): VCAM-1 induction in RHEC-11 by mast cells (contact incubation), showing a distinct percentage of positive cells (D): E-selectin induction in HUVEC by mast cells (contact incubation), also showing a distinct percentage of positive cells.

Mast Cells and ICAM-1 Expression

Confluent monolayers of RHEC-3 were used to study the role of mast cells on the induction of ICAM-1. RHEC-3 showed low basal levels of ICAM-1 expression (about 5% of positive cells) in the absence of mast cells (*data not shown*). In the transwell system, the presence of mast cells resulted in marginally increased expression levels of ICAM-1 in endothelial cells (Table 8.1). Direct contact between mast cells and endothelial cells

promoted the expression of ICAM-1 in RHEC-3 (Table 8.1), but induction levels remained marginal ($6 \pm 1\%$). Both in transwell and direct contact incubations, expression levels of ICAM-1 were not increased in the presence of the mast cell-degranulating agent compound 48/80. In the absence of mast cells, no effects of compound 48/80 as such on ICAM-1 expression were observed.

Table 8.1: Expression of ICAM-1, VCAM-1 and E-selectin in Cultured Endothelial Cells

Incubation ¹	MC ²	Activation ³	Expression (% of positive cells) ⁵					
			ICAM-1	n ⁴	VCAM-1	n	E-selectin	n
Transwell	+	-	2 ± 1	5	17 ± 2	7	6 ± 2	4
Transwell	+	+	2 ± 1	5	19 ± 4	7	6 ± 2	4
Contact	+	-	$6 \pm 1^*$	5	$34 \pm 6^*$	7	$24 \pm 1^*$	4
Contact	+	+	$5 \pm 1^*$	5	$31 \pm 5^*$	7	$26 \pm 5^*$	4
Histamine ⁶	-	-	0	3	0	3	0	2
Cytokines ⁷	-	-	69 (67-71)	2	81 (63-98)	2	54	1
LPS ⁸	-	-	20	1	29	1	71 (63-79)	2

¹ Mast cells were incubated either in direct cell-cell contact (Contact) with endothelial cells, or were separated from these cells by a permeable membrane (Transwell)

² MC(+) indicates the presence of $2.5 \cdot 10^5$ mast cells per incubation (total incubation volume: 2.5 ml)

³ Indicates the presence of the mast cell-degranulating agent compound 48/80 (10 μ g/ml)

⁴ n Indicates the number of experiments

⁵ Results (mean values $\pm$ SEM) are expressed as the percentage of positive cells after correction for basal expression levels in control endothelial cell incubations ($< 5\%$ in all experiments)

⁶ Histamine indicates incubation of endothelial cells in the presence of histamine (35 μ M)

⁷ Cytokines indicates incubation of endothelial cells in the presence of cytokines (a combination of TNF α (400 U/ml) and IFN γ (200 U/ml). The range is indicated between brackets

⁸ LPS indicates incubation of endothelial cells in the presence of lipopolysaccharide (10 μ g/ml)

Asterisks indicate statistically significant differences between expression levels in contact and corresponding transwell incubations

Microscopical examination of the mast cell/EC cultures showed that mast cell degranulation had occurred in compound 48/80-containing incubations. In addition, toluidine blue staining of cytocentrifuge preparations of mast cell/EC incubations with compound 48/80 showed a distinct mast cell degranulation, characterized by the presence of released granules (*not shown*). In cytocentrifuge preparations from mast cell/EC incubations without compound 48/80, mast cell degranulation was almost absent. Mast cell degranulation was also demonstrated by measuring histamine contents in supernatants from mast cell/EC incubations with or without compound 48/80. The histamine content of supernatants from mast cell/EC incubations with compound 48/80 amounted to $80 \pm 5\%$ of the total mast cell histamine content, whereas in the absence of this compound, histamine release was only $4 \pm 2\%$.

The expression of ICAM-1 in RHEC-3 in the presence of mast cells was compared to that achieved upon combined cytokine stimulation. The observation that stimulation with a combination of TNF α and IFN γ resulted in a high percentage of positive cells (71%, mean of two experiments, range 67-75%) indicated that the relatively mild effect

of mast cells with respect to ICAM-1 expression was not due to the fact that RHEC-3 could not adequately be provoked to express this leukocyte-adhesion molecule. This was supported by the finding that also LPS increased expression levels of ICAM-1 (Table 8.1).

Mast Cells and VCAM-1 Expression

The role of mast cells in the expression of VCAM-1 in rat heart endothelial cells was studied in RHEC-11. This cell line showed no basal expression of VCAM-1 (*data not shown*). The expression of VCAM-1 by RHEC-11 was significantly enhanced upon combined cytokine-stimulation with $\text{TNF}\alpha/\text{IFN}\gamma$ (81% of positive cells, mean of two experiments, range 63-98%, Table 8.1). A representative photograph of VCAM-1 expression induced by cytokines is shown in Fig. 8.1B. In the transwell system, incubation of mast cells with endothelial cells induced the expression of VCAM-1, both in the presence or absence of compound 48/80 (Table 8.1). The direct contact of mast cells with endothelial cells increased the expression of VCAM-1 from $17 \pm 2\%$ to $34 \pm 6\%$. Expression levels in these conditions were comparable to those observed upon stimulation with LPS (29%, one experiment). No additional effects of compound 48/80 on expression levels of VCAM-1 were observed ($31 \pm 5\%$). A representative photograph of mast cell-induced VCAM-1 expression in RHEC-11 is shown in Fig. 8.1C. No effects of compound 48/80 as such on VCAM-1 expression were observed. In preliminary experiments, in which the effects of mast cells on the expression of VCAM-1 in HUVEC were studied, effects were comparable to those observed in RHEC-11 (*data not shown*).

Mast cells and E-selectin Expression

Under normal cell-culturing conditions, HUVEC showed a low basal expression (2-3% of positive cells) of E-selectin (*data not shown*). In the transwell system ($n=4$), incubation of mast cells with HUVEC resulted in a small, but significantly increased expression of E-selectin in the latter cells ($6 \pm 2\%$, Table 8.1). Again, no additional effects of compound 48/80 on the expression of E-selectin were observed, whereas large amounts of degranulated mast cells in toluidine blue stained cytocentrifuge preparations were observed. Similar to expression of ICAM-1 and VCAM-1 in cultured rat heart endothelial cells, the direct contact between mast cells and HUVEC strongly promoted the expression of E-selectin in these cells ($24 \pm 1\%$, Table 8.1). Again, no additional effects of compound 48/80 on the expression of E-selectin were observed ($26 \pm 5\%$). In the absence of mast cells, compound 48/80 did not increase basal expression levels of E-selectin (*data not shown*). HUVEC were responsive to stimulation with cytokines or LPS, reaching expression levels of 54% (one experiment) and 75% (mean of two experiments, range 68-79%), respectively. A representative photograph of mast cell-induced E-selectin expression in HUVEC is shown in Fig. 8.1D.

Histamine Stimulation of Endothelial Cells

Although compound 48/80-induced histamine release was not accompanied by an increased expression of endothelial leukocyte-adhesion molecules, this does not rule out the possibility that a low basal release of histamine, observed in all mast cell/EC

incubations, could be responsible for the induction of the investigated endothelial leukocyte-adhesion molecules. Therefore, histamine was added to the endothelial cell incubations in an amount equivalent to the total mast cell histamine content of $2.5 \cdot 10^5$ cells ($35 \mu\text{M}$), or an amount which was ten-fold lower ($3.5 \mu\text{M}$). For both concentrations of histamine tested no effects on expression levels of ICAM-1, VCAM-1 or E-selectin were observed (*data not shown*).

Cardiomyocytes (CMC) and Fibroblasts (CFLC): Stimulation of Endothelial Cells

In order to check if mast cell-induced expression of ICAM-1 and VCAM-1 in rat heart endothelial cells was mast cell-specific, CMC and CFLC were also studied for their capacity to induce expression ICAM-1 and VCAM-1 in RHEC cultures. Neither CMC (at concentrations of $5 \cdot 10^3$ and $5 \cdot 10^4$ cells/incubation) nor CFLC (at a concentration of $5 \cdot 10^5$ cells/incubation) induced ICAM-1 or VCAM-1 in endothelial cells.

8.5 DISCUSSION

We approached the possible role of cardiac mast cells in the induction of endothelial leukocyte-adhesion molecules in the intact heart in a rat experimental model *in vitro*. Peritoneal mast cells were studied for their capacity to increase the expression of ICAM-1, VCAM-1 and E-selectin in cultured endothelial cells. For the effects of mast cells on ICAM-1 and VCAM-1 expression, rat heart endothelial cell (RHEC) lines were used, whereas for E-selectin studies human umbilical vein endothelial cells (HUVEC) were used. In addition, the localization of mast cells in cardiac tissue *in situ* was studied.

In agreement with earlier results of Rakusan *et al.*²⁴, we observed that in the heart *in situ*, mast cells were located around large- and medium-sized blood vessels and around small myocardial vessels, either in the close vicinity of or in contact with endothelial cells. Therefore, in our *in vitro* system, mast cells were allowed to have either direct contact with endothelial cells (*contact incubations*) or were separated from these cells by a permeable membrane (*transwell incubations*).

When compared to expression of ICAM-1 upon stimulation with $\text{TNF}\alpha/\text{IFN}\gamma$, reaching a percentage of positive cells of about 70%, mast cells only marginally induced the expression of ICAM-1 in RHEC-3, both in transwell ($2 \pm 1\%$) and in contact incubations ($6 \pm 1\%$). In contrast, mast cells were found to have profound effects on the expression of VCAM-1 and E-selectin, especially in contact incubations, reaching values of about 40 to 50% of those observed upon cytokine-stimulation. In preliminary studies we observed that the effects of mast cells on VCAM-1 expression in HUVEC were comparable to those observed in RHEC-11. Therefore, it is tempting to assume that the effects of mast cells on the expression of E-selectin in heart endothelial cells would have been similar to those observed in HUVEC. In transwell incubations, mast cells induced the expression of VCAM-1 and E-selectin, but to much lower levels as those found in contact incubations. This suggests that *in vivo*, mast cells may actually stimulate endothelial cells to express leukocyte-adhesion molecules such as VCAM-1 and E-selectin. Since the expression of these adhesion molecules was significantly increased in incubations in which mast cells were in direct contact with endothelial cells, cardiac

mast cells in contact with vascular endothelial cells *in vivo* may be considered most effective in local induction of VCAM-1 and E-selectin. This might be due to the fact that in these conditions, high local concentrations of mast cell-derived mediator(s) are achieved. Moreover, another relevant mechanism may be that 'cross-talk' between mast cells and endothelial cells through direct cell-surface interactions contributes to activation of endothelial cells.

We found that incubation of endothelial cells with mast cells in the presence of compound 48/80 did not have any additional effects on the expression of ICAM-1, VCAM-1 and E-selectin in endothelial cells. The absence of effects of compound 48/80 on adhesion molecule expression was not due to a high extent of non-specific mast cell degranulation, since the histamine content in EC supernatants in non-stimulatory conditions was only $4 \pm 2\%$ of the total mast cell histamine content. In addition, microscopic examination of toluidine blue stained-cytopentrifuge preparations of these incubations revealed a highly homogenous population of mast cells, showing almost no signs of mast cell degranulation. Therefore, the results indicate that (a) mediator(s) responsible for the induction of VCAM-1, E-selectin, and to a lesser extent, ICAM-1, in endothelial cells, is not contained in secretory granules of mast cells, but rather seems to be constitutively released by these cells. For the moment, it cannot be concluded which mediator(s) released from mast cells is (are) responsible for the observed effects. Interestingly, both TNF α and IL-1, which may potentially be released from mast cells¹¹, have been shown to increase the expression of VCAM-1 and E-selectin in endothelial cells *in vitro*.^{3,22} At least for histamine, which is stored in large amounts in secretory granules of mast cells, we could not find any effect of this compound on the induction of ICAM-1, VCAM-1 and E-selectin, both at low and high concentrations. This indicates that histamine can be excluded from the list of putative mast cell-derived products relevant to expression of ICAM-1, VCAM-1 and E-selectin. The observation that cardiomyocytes and fibroblast-like cells did not increase expression levels of ICAM-1 and VCAM-1 suggests that the observed effects are specific for mast cells.

Although the approach used in this *in vitro* study differs at some points from the situation *in vivo* (e.g., absence of blood cells, presence of mast cells at the luminal side of endothelial cells, absence of interstitial matrix structures between mast cells and endothelial cells), the results suggest that *in vivo*, mast cells may be involved in regulation of leukocyte-adhesion during the early inflammatory response. Since continuous expression of E-selectin and VCAM-1 is not observed *in vivo* under normal physiological conditions, this suggests that cardiac mast cells and the, so far, undefined mediator(s) constitutively released from these cells *in vitro*, are apparently little effective in the direct induction of the investigated leukocyte-adhesion molecules *in vivo*. Possibly, (the) mast cell-derived mediator(s) is (are) rapidly degraded or effused, preventing high local concentrations. Alternatively, (the) mediator(s) generated by mast cells may serve to increase the sensitivity of endothelial cells to triggers locally generated by other cells or by the endothelium itself. Another possibility is that *in vivo* products released from other cells residing in the vicinity of mast cells, such as cardiomyocytes, inhibit the formation, release or the effect of the mast cell-derived mediator(s). It is tempting to speculate that after ischemia/reperfusion, in which cardiomyocytes are

severely injured, the capacity of these cells to inhibit the release/formation and/or the effect of mast cell-derived mediators on endothelial cells is reduced or lost. In this context, by increasing the infiltration of leukocytes into the affected region, mast cells might contribute to ischemia/reperfusion-induced injury to cardiomyocytes *in vivo*.

Recent observations have shown that in the rat mesenterium, compound 48/80-induced mast cell degranulation induced P-selectin-dependent leukocyte rolling and CD-18-dependent leukocyte adhesion. These processes were significantly reduced in the presence of the histamine H1-receptor antagonist diphenhydramine and the PAF-antagonist WEB 2086, respectively.¹⁰ In addition, in a similar model, histamine has been shown to induce P-selectin-dependent leukocyte rolling.^{13,14} These results suggest that products released from mast cells, such as histamine, contribute to leukocyte rolling *in vivo*. However, findings from others have indicated that in the rat mesenterium, P-selectin-dependent leukocyte rolling and adhesion in mast cell-dependent inflammation is evoked by mediators other than histamine.³⁰ Therefore, whereas for E-selectin expression we could not find evidence for a role of histamine, for P-selectin-mediated leukocyte rolling the role of histamine is equivocal and, therefore, remains to be established.

In conclusion, data from the present study show that mast cells are capable to induce the expression of VCAM-1, E-selectin and to a lesser extent, ICAM-1 in cultured endothelial cells. The induction of these endothelial leukocyte-adhesion molecules is not related to mast cell degranulation, but seems to be effected by (a) mediator(s) constitutively released from these cells. Direct contact between mast cells and endothelial cells substantially amplifies the expression of these leukocyte-adhesion molecules. Our data indicate that mast cell-derived products other than histamine may play an important role *in vivo* in the concert of early inflammatory events, ultimately leading to the expression of endothelial cell adhesion molecules relevant for infiltration of blood leukocytes.

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CHAPTER 9

General Discussion

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General Remarks

Studies in various cell types, such as endothelial cells and platelets, have indicated that the formation of eicosanoids is strongly influenced by diet-induced modifications in the fatty acid composition of cellular membranes. We were interested to know to which extent in mast cells, the formation of prostanoids and hydroxy fatty acids and the degranulation process were controlled by the membrane fatty acid composition. To study this, a technique to isolate peritoneal mast cells in high purity and a method to quantitate the amount of histamine (a well-known marker of mast cell degranulation) in biological samples were developed. In peritoneal mast cells, it has been shown that a high release of histamine release is not necessarily accompanied by a high release of PGD₂. The responsible mechanisms for this, so-called, differential release of histamine and PGD₂ are largely unknown. We studied to which extent in mast cells, increases in the cytosolic Ca²⁺ concentration and protein tyrosine kinases were responsible for the differential release of histamine and PGD₂. Since studies in tissues such as rat mesenterium and hamster cheek pouch have indicated that products released from mast cells are involved in the expression of leukocyte-adhesion molecules and leukocyte infiltration, peritoneal mast cells were also studied for their capacity to induce the expression of leukocyte-adhesion molecules in rat heart endothelial cells (RHEC). Peritoneal mast cells were used instead of cardiac mast cells, since isolation of the latter cells from the tissue usually results in low yield and high impurity. In the isolated, Langendorff-perfused rat heart, mast cells have been proposed to play a prominent role in acute hypoxia/reoxygenation-induced myocardial injury. However, considerable doubt existed about the techniques used in this study to quantitate the extent of mast cell degranulation. Therefore, we evaluated the role of mast cells in acute hypoxia/reoxygenation-induced injury to cardiomyocytes in this model, using histamine as a marker of mast cell degranulation. The role of mast cells in acute ischemia/reperfusion-induced injury to cardiomyocytes was also studied in the isolated, Langendorff-perfused rat heart.

Peritoneal Mast Cells: Isolation and Quantification of The Degranulation Process

Various techniques for the isolation of mast cells from the peritoneal cavity have been described. However, in most of the techniques, erythrocytes usually contaminate the final mast cell preparation. Since this might result in erroneous data concerning the fatty acid composition of mast cell phospholipids and the production of prostanoids and/or hydroxy fatty acids by mast cells (*Chapter 3*), a method to isolate mast cells in high purity was developed. Therefore, a two-step metrizamide-gradient was introduced into the original procedure of Schwartz *et al.*²⁹ With this technique, peritoneal mast cells could be isolated in high quantity (mean mast cell count of about 8·10⁵ cells per rat) and high purity (>99%). In order to quantitate the extent of mast cell degranulation, a High Performance Liquid Chromatographic (HPLC) method for the determination of histamine was developed. Derivatization of the samples with fluorecamine resulted in samples which were stable over a period of at least a week, which had the advantage that samples needed not be immediately analyzed after derivatization. The method

made use of an ordinary HPLC equipment, including a 'classical' reversed-phase octadecyl (C18) column for the separation of the compounds. At a later stage, the 'classical' reversed-phase HPLC column was replaced by an Inertsil ODS-2 column, which is characterized by a high carbon load and complete end-capping of the residual silanol groups. With this column, elution time, as well as peak tailing and broadening, were significantly reduced.³⁰

Mast Cell Activation and Mediator Release: Role of the Diet

The role of membrane fatty acid composition in the formation of prostanoids and hydroxy fatty acids and in the degranulation process was studied. It was found that feeding rats different dietary fats caused major changes in the relative amounts of fatty acids in mast cell phospholipids. In general, a positive relation was found between the content of (n-6) precursor fatty acids in mast cell phospholipids and A23187-evoked production of prostanoids and hydroxy fatty acids (*Chapter 3*). This is in agreement with results with rat platelets, in which a strict relationship between the level of arachidonic acid in membrane phospholipids and the formation of hydroxyheptadecatrienoic acid (HHT), a marker of thromboxane B₂ formation, was shown.¹¹ Despite considerable changes in the fatty acid composition of membrane phospholipids, no significant changes in compound 48/80-induced histamine release were observed (*Chapter 4*). It was concluded that in mast cells, the fatty acid composition of membrane phospholipids was of importance in those cellular processes in which fatty acids *actively* participated, such as in the formation of prostanoids and hydroxy fatty acids, in which they served as substrates for cyclooxygenase and/or lipoxygenase, respectively. In those cases where fatty acids, as part of phospholipids in mast cell membranes, predominantly served to create a suitable environment for membrane-related processes, such as exocytosis, the fatty acid composition appeared to be less important. Comparable conclusions have drawn for cultured human umbilical vein endothelial cells (HUVEC).^{32,33} In the latter cells, it has been shown that considerable variation in fatty acid composition could be induced in all phospholipid classes by culturing the cells in medium to which specific fatty acids were added. The changes in membrane fatty acid composition strongly influenced the formation of eicosanoids, such as 6-keto-PGF_{1α} and PGF_{2α}, but did not affect membrane-related processes, such as the adherence of polymorphonuclear cells to the endothelial cell monolayer or the shedding of procoagulant microvesicles.^{32,33}

Despite major changes in the pattern of individual fatty acids in mast cell phospholipids, the average *total* amount of saturated (SAFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) was kept within fairly narrow limits (Fig. 9.1). For instance, after feeding a mackerel oil diet, the increased contents of (n-3) fatty acids in mast cell phospholipids, such as timnodonic acid, 20:5(n-3), clupanodonic acid, 22:5(n-3), and cervonic acid, 22:6(n-3), were accompanied by decreased contents of arachidonic acid, 20:4(n-6), and one of its elongation products, 22:4(n-6). In addition, no differences in the unsaturation index of the phospholipid fatty acids¹¹ were observed between the three dietary groups (133.7 ± 4.9 , 130.9 ± 5.6 and 142.2 ± 7.0 in the

HCO, SO or MO-group, respectively). This suggests that in mast cells, the mean number of double bonds, and thus, physico-chemical properties of cellular membranes, are subject to a strong, 'homeostatic control'. Similar conclusions have been reached in HUVEC^{32,33} and, more recently, in rat platelets.¹⁰

Figure 9.1: Global fatty acid composition of total phospholipids in mast cells from rats fed different diets. Mean weight percentages ($n=6$) of saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) are given. HCO: hydrogenated coconut oil; SO: sunflowerseed oil; MO: mackerel oil.

Differential Release of Histamine and PGD₂: Roles of Cytosolic Ca²⁺ and Protein Tyrosine Kinases

The role of the cytosolic Ca²⁺ concentration and protein tyrosine kinases in the differential release of histamine and PGD₂ in mast cells was studied (Chapter 5). Cells were stimulated with the G protein-activating compound 48/80, which by activation of phospholipase C_β (PLC_β), results in the formation of inositol trisphosphate (IP₃) and diacylglycerol (DAG, Fig. 9.2). IP₃ binds to a specific receptor (IP₃R) and results in the release of Ca²⁺ from the internal stores. DAG, together with an increase in [Ca²⁺]_i, stimulates protein kinase C (PKC), which, in turn, phosphorylates various cellular proteins. Mast cells were also incubated in the presence of thapsigargin, which, by its inhibiting action on endomembrane Ca²⁺-ATPases, results in a PLC-independent increase in [Ca²⁺]_i.^{23,28} In addition, mast cells were stimulated with the Ca²⁺ ionophore ionomycin, or the Mg²⁺-free form of ATP, ATP⁴⁻, which, by binding to a specific P_{2y} receptor (R₁), results in opening of receptor-operated channels (ROC).²⁰

The results indicated that in mast cells, as in many other cells such as platelets^{22,28}, the entry of Ca²⁺ across the plasma membrane was coupled to emptying of the intracellular Ca²⁺ stores. The most compelling evidence for this hypothesis came from the observation that inhibition of refilling of the Ca²⁺ stores by thapsigargin resulted in the influx of extracellular Ca²⁺. This notion was in agreement with results from

electrophysiological studies in mast cells, in which depletion of the internal Ca^{2+} stores resulted in specific inward Ca^{2+} currents.^{12,21} The mechanism responsible for this, so-called, capacitive Ca^{2+} influx, is still far from clear. The results suggested that emptying of the internal Ca^{2+} -stores was accompanied by the activation or generation of an unknown factor (Factor X), which was responsible for the influx of Ca^{2+} through store-regulated channels (SRC, Fig. 9.2). Experiments in other cell types have indicated that a small diffusible messenger named CIF (Calcium Influx Factor) or a small G protein might be responsible for the capacitive Ca^{2+} influx.^{3,8,26} In general, the store-regulated Ca^{2+} influx promoted the release of histamine and PGD_2 , but the preference of each agonist to promote the release of histamine or PGD_2 remained unchanged. Therefore, differences in agonist-induced rises in $[\text{Ca}^{2+}]_i$ were considered not to be responsible for the differential release of histamine and PGD_2 in mast cells.

Figure 9.2: Schematic representation of signal transduction pathways in mast cells

SRC: store-regulated channel; ROC: receptor-operated channel; R₁: receptor; G_p: GTP binding protein; PLC β : phospholipase C β ; DAG: diacylglycerol; PKC: protein kinase C; IP₃: inositol (1,4,5) trisphosphate; TykK: protein tyrosine kinase; MAPK: mitogen-activated protein kinase; cPLA₂: cytosolic phospholipase A₂; AA: arachidonic acid; CO: cyclooxygenase; PGDS: prostaglandin D₂ synthase; PGD₂: prostaglandin D₂; IP₃R: inositol (1,4,5) trisphosphate receptor; ER: endoplasmic reticulum

Besides its prominent role in Ca^{2+} influx, the results also indicated that (intracellular signaling events associated with) emptying of the Ca^{2+} stores was required to evoke a substantial release of histamine and PGD_2 . For instance, stimulation of mast cells with

CaCl_2 and ATP^{4-} , or the addition of CaCl_2 to store-depleted cells, resulted in a high increase in $[\text{Ca}^{2+}]_i$, but in the release of only minor quantities of histamine and PGD_2 . Typically, in these cases, the increase in $[\text{Ca}^{2+}]_i$ was solely evoked by the influx of extracellular Ca^{2+} and not by emptying of the internal Ca^{2+} stores. In contrast, in those conditions in which Ca^{2+} responses consisted of Ca^{2+} influx in combination with store-emptying, e.g., after stimulation of the cells with compound 48/80, thapsigargin or ionomycin, a substantial release of histamine and PGD_2 was observed. In platelets, it has been shown that the increase in $[\text{Ca}^{2+}]_i$ caused by emptying of the internal Ca^{2+} stores, played a prominent role in tyrosine phosphorylation of specific cellular proteins, which, in turn, might regulate plasma membrane permeability for Ca^{2+} .³⁴ We found that protein tyrosine kinases were involved in both the release of histamine and PGD_2 . Hence, it is possible that in mast cells, emptying of the Ca^{2+} stores is related to activation of protein tyrosine kinases, which may explain the effects of inhibitors of these kinases on the release of histamine and PGD_2 . The exact mechanisms by which protein tyrosine kinases in mast cells are activated are largely unknown, and, therefore remain to be clarified.

Although we did not measure protein phosphorylation patterns directly, our data suggested that in mast cells, protein tyrosine kinases were more prominently involved in the process of eicosanoid formation than in exocytosis. Experiments with store-depleted mast cells indicated that, besides protein tyrosine kinases, prolonged high levels of $[\text{Ca}^{2+}]_i$ might also play an important role in promoting the formation of PGD_2 . The high histamine secretion evoked by compound 48/80, is known to be due to the activation of protein kinase C^9 ,²¹ and/or the newly identified protein, G_{i3} .² Judging from the results in other cell types, protein tyrosine kinases might play an important role in activation of mitogen-activated kinases (MAP kinases)^{15,18}, which, together with an increase in $[\text{Ca}^{2+}]_i$,^{4,5} result in activation of cytosolic phospholipase A_2 (cPLA₂). This enzyme has been shown to be responsible for agonist-evoked release of arachidonic acid (AA) in most cell types.^{15,17}

Mast Cells and Injury to Cardiomyocytes

Results from *in vivo* and *ex vivo* studies have indicated that mast cells play a role in ischemia/reperfusion or hypoxia/reoxygenation-induced myocardial injury.^{13,14} Much of this evidence is based on the stabilizing effects of Iodoxamide on myocardial mast cells. In order to study the role of mast cells in ischemia/reperfusion-induced injury to cardiomyocytes in a more direct way, hearts, isolated from sensitized and non-sensitized rats³¹, were perfused according to Langendorff. Previously, it has been shown that antigen-challenge resulted in a massive mast cell degranulation in sensitized hearts³¹, a phenomenon which is absent in non-sensitized, control hearts. 'Mast cell-depleted' and control hearts were subjected to short-term ischemia/reperfusion. It was found that during normoxic perfusion, a massive mast cell degranulation was not followed by an increased release of lactate dehydrogenase (LDH) and, thus, did not result in (irreversible) injury to cardiomyocytes. During reperfusion, LDH release in previously ischemic control hearts was equal to that in 'mast cell-depleted' hearts. Therefore, the

results provided no evidence that mast cells were prominently involved in *acute* ischemia/reperfusion-induced injury to cardiac muscle cells. In a hypoxia/reoxygenation model of myocardial injury, it was found that the release of histamine, used as a marker of mast cell degranulation, did not correlate to that of LDH or creatine kinase (CK). In addition, no correlation between the release of histamine and that of peroxidase (PO), a proposed marker of mast cell degranulation¹⁴, was observed. Determination of PO-activity in isolated cardiomyocytes revealed that in the rat heart, the bulk of PO-activity was located in these cells. The results showed that the conclusion of Keller *et al.*¹⁴ on the role of mast cells in acute hypoxia/reoxygenation-induced injury to cardiomyocytes, was based on the incorrect use of PO as a marker of mast cell degranulation. In fact, the results indicated that in the isolated rat heart, mast cell degranulation was not related to the extent of injury to cardiomyocytes, both in the case of ischemia/reperfusion, when this injury was relatively low (*Chapter 6*) and in hypoxia/reoxygenation, when the injury was high (*Chapter 7*). Although the models differed at some points from the situation *in vivo* (no blood cells, no innervation), it was concluded that there was no evidence that *in vivo* products released from degranulated mast cells were directly involved in *acute* ischemia/reperfusion-induced injury to cardiomyocytes.

Mast cells and the Induction of Endothelial Leukocyte-Adhesion Molecules

Data from *in vitro* studies in which rat heart endothelial cells were used (*Chapter 8*), indicated that *longer-term* effects of mast cell-derived products might include the infiltration of leukocytes into cardiac tissue. It was found that both under conditions in which mast cells were in direct contact with endothelial cells (contact studies) and in conditions in which they were separated from endothelial cells through a permeable membrane (transwell studies), products released from mast cells increased the expression of VCAM-1 and E-selectin, and to a lesser extent, ICAM-1, by endothelial cells. These molecules play a prominent role in the adhesion of leukocytes to the vascular endothelium, a necessary first-step in leukocyte migration.¹ The expression of VCAM-1 and E-selectin was highest when mast cells were in direct contact with endothelial cells. This suggested that in these conditions high local concentrations of mast cell-derived mediators were achieved. However, it could not be excluded that direct 'cell-cell' interactions between mast cells and endothelial cells were responsible for (part of) the observed effects. The expression of VCAM-1 and E-selectin by endothelial cells was not related to mast cell degranulation, which indicated that the expression of these leukocyte-adhesion molecules was evoked by (a) mediator(s) constitutively released from mast cells. The nature of this (these) mediator(s) is unknown, but the results indicated that at least histamine could be excluded from the list of putative mast cell-derived products. Since, under normal physiological conditions *in vivo*, continuous expression of E-selectin and VCAM-1 is not observed, the responsible mast cell-derived mediator(s) was (were) considered to be little effective in the direct induction of these endothelial leukocyte-adhesion molecules. Possibly, *in vivo*, the mediator(s) is (are) rapidly degraded or effused, preventing high local concentrations. Alternatively, it was proposed that the mast cell-derived mediator(s) might serve to

increase the sensitivity of endothelial cells to triggers locally generated by cells other than mast cells or by the endothelium itself. Another possibility is that *in vivo*, products released from cells residing in the vicinity of mast cells, such as cardiomyocytes, inhibit the formation and/or release or the effects of mast cell-derived mediator(s). In this context, it was speculated that during ischemia/reperfusion, in which the inhibitory effects of cardiac muscle cells on mast cells might be decreased or lost, the release of mast cell-derived mediators could contribute to the infiltration of leukocytes into cardiac tissue by promoting the expression of VCAM-1 and E-selectin. Since the infiltration of leukocytes after ischemia/reperfusion is closely related to the extent of myocardial injury^{7,16,27}, it is tempting to speculate that mast cells contribute to this kind of injury by increasing the expression of endothelial leukocyte-adhesion molecules. Besides negative effects of mast cells in longer-term injury in the heart, it cannot be excluded that mast cells also exert positive effects. For instance, it has been shown that in a model of prolonged ischemia (3 weeks), mast cells accumulate in the subepicardial layer of the infarcted region.⁶ Since these cells are prominently involved in angiogenesis^{19,24,25}, this may be of importance in prevention of further irreversible injury to myocardial tissue.

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In this thesis, the role of short- and long-term memory in the generation-induced response of cultured Langerhans cells to antigen presentation and mitogenic stimulation and the effects of extracellular Ca^{2+} concentration, pH and proteolysis on the response of these cells are studied.

Some general aspects of immunology are reviewed in chapter 1. Multicellular organisms are able to respond to a wide variety of antigens. Antigen presentation is a process by which a small fragment of an antigen is presented to a T cell by a major histocompatibility complex (MHC) molecule. The MHC is a polymorphic gene complex that encodes the antigen binding site. The MHC is divided into two main regions, the class II region and the class III region. The class II region encodes the MHC molecules that present antigens to T cells. The class III region encodes the MHC molecules that present antigens to B cells. The class I region encodes the MHC molecules that present antigens to natural killer (NK) cells. The class II region is further divided into two main regions, the class IIa region and the class IIb region. The class IIa region encodes the MHC molecules that present antigens to T cells. The class IIb region encodes the MHC molecules that present antigens to B cells. The class I region encodes the MHC molecules that present antigens to NK cells. The class II region is further divided into two main regions, the class IIa region and the class IIb region. The class IIa region encodes the MHC molecules that present antigens to T cells. The class IIb region encodes the MHC molecules that present antigens to B cells. The class I region encodes the MHC molecules that present antigens to NK cells.

SUMMARY

Antigen presentation and hypocalcaemia-induced release of histamine from mast cells. This was studied in the mast cells of the rat. The specific aspects of mast cell activation and histamine release were studied in particular. The role of extracellular Ca^{2+} concentration, pH and proteolysis on the response of these cells are studied. The role of short- and long-term memory in the generation-induced response of cultured Langerhans cells to antigen presentation and mitogenic stimulation and the effects of extracellular Ca^{2+} concentration, pH and proteolysis on the response of these cells are studied. The role of short- and long-term memory in the generation-induced response of cultured Langerhans cells to antigen presentation and mitogenic stimulation and the effects of extracellular Ca^{2+} concentration, pH and proteolysis on the response of these cells are studied. The role of short- and long-term memory in the generation-induced response of cultured Langerhans cells to antigen presentation and mitogenic stimulation and the effects of extracellular Ca^{2+} concentration, pH and proteolysis on the response of these cells are studied.

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In this thesis, the role of mast cells in acute ischemia/reperfusion and hypoxia/reoxygenation-induced injury to cardiomyocytes is described. This was studied in the isolated, Langendorff-perfused rat heart. In addition, various specific aspects of mast cell activation and mediator release, such as the roles of membrane fatty acid composition, the cytosolic Ca^{2+} concentration and protein tyrosine kinases in the release of histamine and prostanoids, are described. The latter aspects were studied in peritoneal mast cells.

Some general aspects of mast cell activation and mediator release are discussed in **Chapter 1**. Mast cells are inflammatory cells found in various tissues such as lung, heart and skin. Mediators released from these cells play an important role in the clinical signs of hay-fever and asthma. In their secretory granules, mast cells contain a large variety of mediators, such as histamine, which are rapidly released upon activation. In addition, mast cells release a variety of *newly formed* mediators such as prostanoids and hydroxy fatty acids. In order to quantitate the extent of mast cell degranulation, a rapid and highly sensitive High Performance Liquid Chromatographic (HPLC) method for the determination of histamine in biological samples was developed. This technique was used to determine the amounts of histamine in cardiac tissue, coronary effluents and supernatants of stimulated peritoneal mast cells. Since in most of the techniques for the isolation of peritoneal mast cells described until now, erythrocytes contaminate the final mast cell preparation, a method was developed to isolate these cells from the peritoneal cavity in high purity. This method and the HPLC procedure for the determination of histamine are described in **Chapter 2**.

Certain fatty acids incorporated in membrane phospholipids, such as linoleic or arachidonic acid, serve as precursors for the synthesis of prostanoids and hydroxy fatty acids. We were interested to know to which extent diet-induced changes in mast cell membrane fatty acid composition influenced the formation of prostanoids and hydroxy fatty acids. Therefore, peritoneal mast cells were isolated from rats fed diets which differed in their fatty acid composition (**Chapter 3**). It was found that the formation of prostanoids and hydroxy fatty acids was strongly influenced by diet-induced changes in mast cell phospholipid fatty acid composition. We hypothesized that diet-induced changes in mast cell phospholipid fatty acid composition might influence membrane-related processes such as exocytosis (**Chapter 4**). Although the different dietary fats induced major changes in the fatty acid composition of mast cell phospholipids, no differences in compound 48/80-induced histamine release were observed. It was concluded that the extent of mast cell degranulation was independent of the fatty acid composition of membrane phospholipids.

In **Chapter 5**, the role of the cytosolic Ca^{2+} concentration and protein tyrosine kinases in the differential release of histamine and PGD_2 in mast cells is described. Mast cells were stimulated with the G protein-activating compound 48/80, with thapsigargin, an inhibitor of endomembrane Ca^{2+} -ATPases, with the Ca^{2+} ionophore ionomycin or with the Mg^{2+} -free form of ATP, ATP^{4-} . It was found that stimulation of the cells with compound 48/80 evoked a high histamine secretion and a low PGD_2 release, whereas the Ca^{2+} mobilizers thapsigargin and ionomycin evoked a low histamine secretion and a high release of PGD_2 . Despite prolonged high levels of $[\text{Ca}^{2+}]_i$, ATP^{4-} did not evoke a

substantial release of histamine or PGD₂. In the absence of extracellular CaCl₂, *i.e.*, in those cases in which the influx of extracellular Ca²⁺ was absent, agonist-induced rises in [Ca²⁺]_i as well as histamine secretion and PGD₂ release were significantly reduced. In addition, it was found that the release of histamine was much more influenced by inhibitors of protein tyrosine kinases than the release of PGD₂. It was concluded that the store-regulated influx of extracellular Ca²⁺ amplified agonist-induced rises in [Ca²⁺]_i as well as histamine secretion and PGD₂ release. The differential release of histamine and PGD₂ appeared not to be due to differences in the increase in [Ca²⁺]_i, but rather to a different involvement of protein tyrosine kinases in both processes.

The heart has been shown to contain a resident population of mast cells of which the (patho-)physiological role is largely unknown. It has been suggested that in the heart *ex vivo*, mast cells play a role in hypoxia/reoxygenation-induced injury to cardiac muscle cells. However, there is considerable doubt about the marker used to quantitate the extent of mast cell degranulation. Mast cells have also been proposed to play a role in ischemia/reperfusion-induced myocardial injury *in vivo*. We firstly studied to which extent mast cells were involved in *acute* ischemia/reperfusion-induced injury to cardiomyocytes (**Chapter 6**). Therefore, hearts isolated from non-sensitized, control rats or sensitized rats, were perfused according to Langendorff. During normoxic perfusion, hearts were challenged with antigen, a procedure which is known result in a massive mast cell degranulation in sensitized hearts. After 20 min, hearts were subjected to 30 min of global ischemia followed by 30 min of reperfusion. Since injury to cardiomyocytes results in a rapid leakage of lactate dehydrogenase (LDH), the activity of this enzyme was determined in coronary effluents in order to quantitate the extent of irreversible damage to these cells. During reperfusion, no differences in LDH release between control hearts and 'mast cell-depleted hearts' were found. Thus, the results provided no evidence that in this model, mast cells were prominently involved in *acute* ischemia/reperfusion-induced injury to cardiomyocytes.

The role of mast cells in *acute* hypoxia/reoxygenation-induced injury to cardiomyocytes was studied using histamine as a marker of mast cell degranulation (**Chapter 7**). Isolated, Langendorff-perfused rat hearts were subjected to 35 min of normoxic perfusion, followed by 60 min of hypoxic perfusion and 30 min of reoxygenation. The activities of LDH or creatine kinase (CK) were determined in coronary effluents in order to quantitate the extent of (irreversible) injury cardiomyocytes. Special attention was paid to the suitability of peroxidase (PO) as a marker of mast cell degranulation. During reoxygenation, the release of LDH, CK and PO did not correlate to that of histamine. When hearts were perfused in the presence of the mast cell stabilizer Iodoxamide, histamine release during reoxygenation significantly decreased, whereas the release of LDH, CK or PO remained unchanged. Determination of PO-activity in isolated cardiomyocytes learned that the bulk of PO-activity in the heart was located in these cells and not in mast cells. Therefore, it was concluded that in the isolated rat heart, PO release was not a suitable marker to indicate the extent of mast cell degranulation. In addition, the results provided no evidence that in this model, mast cells were prominently involved in the extent of *acute* hypoxia/reoxygenation-induced injury to cardiomyocytes.

In tissues such as rat mesenterium and hamster cheek pouch, products released from mast cells have been found to play a prominent role in the expression of endothelial leukocyte-adhesion molecules and leukocyte infiltration. In the process of leukocyte-adhesion, the interaction between endothelial leukocyte-adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin with their respective counterparts on circulating leukocytes is an important step. Until now, information about the role of mast cells in the adhesion of leukocytes to cardiac endothelium is lacking. The role of mast cells in the induction of endothelial leukocyte-adhesion molecules in the heart *in vivo* was approached in an *in vitro* rat experimental model (**Chapter 8**). Peritoneal mast cells were studied for their capacity to induce the expression of ICAM-1, VCAM-1 and E-selectin in cultured endothelial cells. For ICAM-1 and VCAM-1 studies, rat heart endothelial cell (RHEC) lines were used. For E-selectin studies, human umbilical vein endothelial cells (HUVEC) were used, since no suitable rat E-selectin antibodies for the rat system were available. Mast cells were incubated either in direct cell-cell contact with endothelial cells or were separated from these cells through a permeable membrane. It was found that in both conditions, products released from mast cells increased the expression of VCAM-1 and E-selectin, and to a minor extent, that of ICAM-1 in endothelial cells. The direct contact between mast cells and endothelial cells significantly promoted the expression of these leukocyte-adhesion molecules. It was concluded that the induction of ICAM-1, VCAM-1 and E-selectin by endothelial cells was not related to the extent of mast cell degranulation, but seemed to be evoked by the release of a(n), so far, undefined substance(s) other than histamine, constitutively released from mast cells.

In **Chapter 9** the results described in this thesis are summarized and discussed in a broader perspective.

| SAMENVATTING

In dit proefschrift wordt de rol van mestcellen in acute ischemie/reperfusie en hypoxie/reoxygenatie-geïnduceerde schade aan hartspiercellen beschreven. Dit werd bestudeerd in het geïsoleerde, Langendorff-geperfundeerde rattehart. Tevens worden enkele aspecten van mestcel-activatie en het vrijstellen van mediators beschreven. Met name op de rol van de vetzuursamenstelling van het celmembran, de intracellulaire calcium concentratie en tyrosine kinases in de vrijstelling van histamine en prostanoïden wordt nader ingegaan. Deze laatste aspecten werden bestudeerd in peritoneale mestcellen.

Enkele algemene aspecten van mestcellen en het vrijstellen van mediators worden beschreven in **Hoofdstuk 1**. Mestcellen zijn ontstekingscellen die in verschillende weefsels, zoals long, hart en huid worden aangetroffen. Mediators die door deze cellen vrijgesteld worden, spelen een belangrijke rol in de klinische verschijnselen van hooikoorts en astma. In de granules van deze cellen bevinden zich vele mediators, zoals histamine, die snel vrijgesteld kunnen worden als de cel wordt geactiveerd. Tevens kunnen mestcellen een aantal mediators vrijstellen, die *nieuw* door de cel worden gemaakt, zoals bijvoorbeeld de prostanoïden en hydroxyvetzuren. Om de mate van mestceldegranulatie te kwantificeren, werd een methode ontwikkeld om de hoeveelheid histamine in biologische monsters te bepalen. Deze methode, die gebruik maakt van hoge druk vloeistofchromatografie (HPLC), werd gebruikt om de hoeveelheid histamine in hartspierweefsel, coronaire effluents en supernatanten van gestimuleerde mestcellen te bepalen. Omdat in de meeste methodes voor de isolatie van peritoneale mestcellen die tot nu beschreven zijn, erythrocyten meestal in het uiteindelijke mestcelpreparaat aanwezig zijn, werd een methode ontwikkeld om mestcellen in hoge zuiverheid uit de buikholte te isoleren. Deze procedure en de HPLC methode voor de bepaling van histamine worden beschreven in **Hoofdstuk 2**.

Bepaalde vetzuren die ingebouwd zijn in membraanfosfolipiden, zoals linolzuur of arachidonzuur, kunnen worden gebruikt voor de synthese van prostanoïden en hydroxyvetzuren. Wij onderzochten in hoeverre de vorming van prostanoïden en hydroxyvetzuren in mestcellen beïnvloed werd door dieet-geïnduceerde veranderingen in de vetzuursamenstelling van membraanfosfolipiden. Daarom werden peritoneale mestcellen geïsoleerd uit ratten die gevoed waren met diëten van een verschillende vetzuursamenstelling (**Hoofdstuk 3**). Het bleek dat de vorming van prostanoïden en hydroxyvetzuren in mestcellen sterk beïnvloed werd door dieet-geïnduceerde veranderingen in de vetzuursamenstelling van membraanfosfolipiden. In **Hoofdstuk 4** wordt de rol van de vetzuursamenstelling van membraanfosfolipiden in de mate van mestceldegranulatie beschreven. Ondanks dat de verschillende dieetvetten belangrijke veranderingen in de vetzuursamenstelling van mestcelfosfolipiden induceerden, werden er geen belangrijke veranderingen waargenomen in de mate van compound 48/80-geïnduceerde vrijstelling van histamine. Daarom werd geconcludeerd dat de mate van mestceldegranulatie onafhankelijk was van de vetzuursamenstelling van membraanfosfolipiden.

In **Hoofdstuk 5** wordt de rol van de intracellulaire Ca^{2+} concentratie en tyrosine kinases in de differentiële vrijstelling van histamine en PGD_2 in mestcellen beschreven. Mestcellen werden gestimuleerd met het G eiwit-activerende compound 48/80, met

thapsigargine, een remmer van Ca^{2+} -ATPases, met de Ca^{2+} ionofoor ionomycine of met de Mg^{2+} -vrije vorm van ATP, ATP^{4-} . Activatie met compound 48/80 resulteerde in een hoge vrijstelling van histamine en een lage vrijstelling van PGD_2 , terwijl activatie met thapsigargine of ionomycine resulteerde in een lage vrijstelling van histamine en een relatief hoge vrijstelling van PGD_2 . Ondanks langdurig hoge intracellulaire Ca^{2+} concentraties ($[\text{Ca}^{2+}]_i$), resulteerde incubatie met ATP^{4-} niet in een substantiële vrijstelling van histamine of PGD_2 . In de afwezigheid van extracellulair CaCl_2 (= in de afwezigheid van influx van extracellulair Ca^{2+}), daalden de agonist-geïnduceerde toenames in $[\text{Ca}^{2+}]_i$ en de vrijstelling van histamine en PGD_2 . Het bleek dat de vrijstelling van PGD_2 veel sterker beïnvloed werd door remmers van tyrosine kinases dan de vrijstelling van histamine. Er werd geconcludeerd dat de influx van extracellulair Ca^{2+} zowel de agonist-geïnduceerde stijgingen van $[\text{Ca}^{2+}]_i$ als de vrijstelling van histamine en PGD_2 verhoogde. De differentiële vrijstelling van histamine en PGD_2 bleek niet afhankelijk van verschillende stijgingen van $[\text{Ca}^{2+}]_i$, maar eerder van een verschillende rol van tyrosine kinases in beide processen.

Het hart bevat een residente populatie van mestcellen, waarvan de pathofysiologische rol vrijwel onbekend is. Resultaten van een *ex vivo* studie hebben gesuggereerd dat mestcellen een rol zouden kunnen spelen in acute hypoxie/reoxygenatie-geïnduceerde schade aan hartspiercellen. Er bestaat echter grote twijfel over de maat die gebruikt werd om de mate van mestceldegranulatie te kwantificeren. Er zijn aanwijzingen dat mestcellen ook een rol spelen in ischemie/reperfusie-geïnduceerde hartspierschade *in vivo*. Wij hebben allereerst bestudeerd in hoeverre mestcellen een rol spelen in acute ischemie/reperfusie-geïnduceerde schade aan hartspiercellen (**Hoofdstuk 6**). Harten die geïsoleerd waren uit niet-gesensitiseerde, controle ratten of uit gesensitiseerde ratten werden geperfundeed volgens Langendorff. Gedurende de normoxische perfusie werd aan alle harten antigeen toegediend, waarvan bekend is dat het leidt tot een massale mestceldegranulatie in gesensitiseerde harten. Na 20 min normoxische perfusie werden alle harten onderworpen aan 30 min ischemie gevolgd door 30 min reperfusie. Omdat schade aan hartspiercellen leidt tot het lekken van lactaat dehydrogenase (LDH), werd de activiteit van dit enzym bepaald in coronaire effluenten om de mate van irreversibele schade aan deze cellen te kwantificeren. Tijdens de reperfusie-fase werden geen verschillen waargenomen in de mate van LDH-vrijstelling tussen de controle en de gesensitiseerde harten. De resultaten leverden geen bewijs dat mestcellen in dit model een belangrijke rol speelden in de mate van *acute* ischemie/reperfusie-geïnduceerde schade aan hartspiercellen.

De rol van mestcellen in acute hypoxie/reoxygenatie-geïnduceerde schade aan hartspiercellen werd bestudeerd door histamine als maat voor mestceldegranulatie te gebruiken (**Hoofdstuk 7**). Geïsoleerde, Langendorff-geperfundeerde harten werden onderworpen aan 35 minuten normoxische perfusie, gevolgd door 60 minuten hypoxische perfusie en 30 minuten reoxygenatie. De activiteiten van LDH en creatine kinase (CK) werden bepaald in coronaire effluenten om de mate van irreversibele schade aan hartspiercellen te kwantificeren. Speciale aandacht werd besteed aan de geschiktheid van peroxidase (PO) als maat voor mestceldegranulatie. Gedurende de

reoxxygenatie-fase werd geen correlatie gevonden tussen de vrijstelling van LDH, CK en PO, en de mate van histamine vrijstelling. Wanneer harten geperfundeed werden in de aanwezigheid van de mestcelstabilisator Iodoxamide, daalde gedurende de reoxxygenatie-fase de vrijstelling van histamine, maar de vrijstelling van LDH, CK en PO bleef onveranderd. Bepaling van de PO-aktiviteit in geïsoleerde hartspiercellen toonde aan de meerderheid van PO-aktiviteit in het hart gelokaliseerd was in deze cellen en *niet* in mestcellen. Daarom werd geconcludeerd dat de vrijstelling van PO in dit model geen geschikte maat was om de mate van mestceldegranulatie te bepalen. Tevens leverden deze resultaten geen bewijs dat mestcellen een belangrijke rol spelen in de mate van *acute* hypoxie/reoxxygenatie-geïnduceerde schade aan hartspiercellen.

In weefsels, zoals het mesenterium van de rat of de hamster-wangzak, is het aangetoond, dat mediators die vrijgesteld worden door mestcellen betrokken zijn in de expressie van leukocyt-adhesie moleculen en leukocyt-infiltratie. In het proces van leukocyt-adhesie speelt de interactie tussen endotheliale leukocyt-adhesie moleculen, zoals ICAM-1, VCAM-1 en E-selectine met hun respectievelijke liganden op circulerende leukocyten, een belangrijke rol. Tot nu toe is er niets bekend over de rol van mestcellen in de adhesie van leukocyten aan hart endotheel. De rol van mestcellen in de inductie van endotheliale leukocyt-adhesie moleculen *in vivo*, werd bestudeerd in een *in vitro* rat experimenteel model (**Hoofdstuk 8**). Daartoe werd gekeken in hoeverre peritoneale mestcellen in staat waren om de expressie van ICAM-1, VCAM-1 of E-selectine op gekweekte endotheelcellen te induceren. Voor ICAM-1 en VCAM-1 studies werden lijnen van rattehart endotheelcellen (RHEC) gebruikt, terwijl voor de E-selectine studies humane navelstreng endotheelcellen (HUVEC) werden gebruikt, omdat E-selectine antilichamen voor het rat-systeem niet beschikbaar waren. Mestcellen werden geïncubeerd in direct 'cel-cel' contact met endotheelcellen *of* ze werden van deze cellen gescheiden door middel van een permeabel membraan. Het bleek dat in beide condities, stoffen die door mestcellen werden vrijgesteld, de expressie van VCAM-1 en E-selectine, en in mindere mate van ICAM-1, op endotheelcellen konden verhogen. Het directe contact tussen mestcellen en endotheelcellen veroorzaakte een significante stijging van de expressie van deze leukocyt-adhesie moleculen. De inductie van ICAM-1, VCAM-1 en E-selectine was niet gerelateerd aan de mate van mestceldegranulatie, maar bleek veroorzaakt te worden door (een) stof(fen) anders dan histamine, die constitutief door mestcellen werd(en) vrijgesteld.

In **Hoofdstuk 9** worden de resultaten zoals beschreven in dit proefschrift samengevat en in een breder kader geplaatst.

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De auteur van dit proefschrift werd geboren op 13 juli 1967 te Maastricht. Na het behalen van het VWO-B diploma aan het Sint Maartenscollege te Maastricht in juni 1986, werd in hetzelfde jaar begonnen met de studie Gezondheidswetenschappen, afstudeerrichting Biologische Gezondheidkunde, aan de Rijksuniversiteit Limburg te Maastricht. Na de afstudeerstage via het Erasmus studenten-uitwisselingsproject aan het Klinisch Chemisch Laboratorium van het AZ Sint-Jan te Brugge (B) en een stage op de afdeling Algemene Biochemie van Janssen Pharmaceutica te Beerse (B), behaalde hij in maart 1991 het doctoraal examen. Van mei 1991 tot mei 1995 was hij werkzaam als Assistent In Opleiding aan het Instituut Hart- en Vaatziekten aan de Rijksuniversiteit Limburg te Maastricht, alwaar hij de werkzaamheden zoals beschreven in dit proefschrift uitvoerde.

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