From the Department of Dental Medicine
Karolinska Institutet, Stockholm, Sweden

Prostaglandin E synthases in periodontitis-affected gingival tissue and in gingival fibroblasts

Tove Båge

Stockholm 2011

Cover illustration, clockwise from top left:
Periodontitis-affected gingival tissue, stained for fibroblasts (green) and for mPGES-1 (red).
Exchanging cell culture medium in the sterile hood.
Hybridised microarray chip.
Human gingival fibroblasts in cell culture.

Published by Karolinska Institutet.

Printed by US-AB
Nanna Svartz väg 4
SE-171 77 Solna
Sweden

ISBN 978-91-7457-392-3

“Det heter inte improvisera, det heter forska...”

Ur Magnus och Brasses sketch ”Kirurgerna”

Abstract

Periodontitis is a chronic inflammatory disease resulting in the destruction of the tissue and alveolar bone supporting the teeth and leading ultimately to tooth loss. Prostaglandin E_2 (PGE_2) is an important inflammatory mediator in the pathogenesis of periodontitis. The biosynthesis of PGE_2 is catalysed by three groups of enzymes acting sequentially: phospholipase A_2 (PLA_2), cyclooxygenases (COX-1 and COX-2) and prostaglandin E (PGE) synthases, which catalyse the final step of PGE_2 synthesis. Three PGE synthase isoforms have been identified: i) the inducible microsomal membrane-associated and glutathione-dependent PGE synthase, mPGES-1, ii) the constitutively expressed cytosolic PGE synthase, cPGES, and iii) the glutathione-independent, membrane-associated mPGES-2. The aim of this thesis was to investigate the expression of PGE synthases in gingival tissue from periodontitis patients, as well as to study their expression and regulation in relation to PGE_2 production in gingival fibroblasts.

In periodontitis-affected gingival tissue, we demonstrated *in vivo* protein expression of mPGES-1, mPGES-2 and cPGES, as well as COX-2 in fibroblasts, endothelial cells, smooth muscle cells, epithelial cells and immune cells. We further showed that, in cell cultures of gingival fibroblasts and smooth muscle cells, the inflammatory cytokines tumour necrosis factor α (TNFα) and interleukin-1β (IL-1β), or co-culture with lymphocytes, markedly induced mPGES-1 and COX-2 expression, accompanied by an increase in PGE_2 production. In cultured endothelial cells, only TNFα was found to increase PGE_2 production, via enhanced COX-2 expression. In mast cell cultures, basal levels of PGE_2 were detected, but no increase was observed in response to TNFα or IL-1β.

To elucidate the impact of mPGES-1 inhibition on mPGES-2 and cPGES expression, as well as on PGE_2 production we used knock-down of mPGES-1 expression by small interfering RNA (siRNA). The cytokine-induced protein expression of mPGES-1 was reduced by up to 79% by siRNA silencing, without affecting mPGES-2 or cPGES expression. Moreover, mPGES-1 siRNA did not affect the cytokine-stimulated PGE_2 production, whereas levels of the downstream prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were enhanced.

Using inhibitors and activators of various signalling pathways, we demonstrated that cytokine-induced mPGES-1 expression in gingival fibroblasts did not involve protein kinase C, p38 mitogen-activated protein kinase or tyrosine kinase pathways, in contrast to COX-2 expression. We further observed a possible positive feedback loop in which PGE_2 and $PGF_{2\alpha}$ increased the expression of mPGES-1. Furthermore, cytokine-induced mPGES-1 expression and PGE_2 production were reduced after the inhibition of the upstream enzyme PLA_2 and increased after the addition of arachidonic acid, the product of PLA_2. The proposed anti-inflammatory prostaglandin 15-deoxy-Δ12,14-prostaglandin J_2 (15d-PGJ_2), reduced mPGES-1 expression but not COX-2 expression or PGE_2 production.

To further explore the pathways involved in increased PGE_2 synthesis in TNFα-stimulated gingival fibroblasts, a global gene expression profile was established using a microarray platform. Enrichment analysis of the gene expression data led to further investigation of nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK) signalling pathways, revealing that these pathways are involved in the signal transduction of TNFα-induced mPGES-1 and COX-2 expression.

In conclusion, all three PGE synthases are expressed in gingival tissue from patients with periodontitis. The isoenzyme mPGES-1 is the main PGE synthase involved in

cytokine-induced PGE_2 production in gingival fibroblasts. The cytokine-increased expression of mPGES-1 involves the signal pathways JNK and NF-κB. Furthermore, the prostaglandins PGE_2 and $PGF_{2\alpha}$ increase mPGES-1 expression, which may create a positive feedback loop. Collectively, these results suggest that inflammation-induced production of PGE_2 by gingival fibroblasts, mediated by the increased expression of mPGES-1 and COX-2, may contribute to chronic inflammation in periodontitis. The results provide new insights into the expression and regulation of mPGES-1 in gingival fibroblasts and gingival tissue.

Keywords: c-Jun N-terminal kinase, cyclooxygenase, cytokines, gingival fibroblasts, gingival tissue, inflammation, interleukin-1β, nuclear factor-κB, mPGES-1, periodontitis, prostaglandin E_2, prostaglandin E synthase, tumour necrosis factor α

List of publications

I. **Tove Båge***, Anna Kats*, Blanca Silva Lopez, Gareth Morgan, Gunnar Nilsson, Idil Burt, Marina Korotkova, Lisa Corbett, Alan J. Knox, Leonardo Pino, Per-Johan Jakobsson, Thomas Modéer, Tülay Yucel-Lindberg. Expression of prostaglandin E synthases in periodontitis: immunolocalization and cellular regulation. *Am J Pathol* 2011 Apr; 178(4): 1676-88.
*These authors contributed equally to this work.

II. **Tove Båge**, Thomas Modéer, Tomomi Kawakami, Hernan Concha Quezada, Tülay Yucel-Lindberg. Regulation of prostaglandin E synthases: Effects of siRNA-mediated inhibition of microsomal prostaglandin E synthase-1. *Biochim Biophys Acta – Mol Cell Res* 2007 Oct; 1773(10): 1589-98.

III. Tülay Yucel-Lindberg*, **Tove Olsson***, Tomomi Kawakami. Signal pathways involved in the regulation of prostaglandin E synthase in human gingival fibroblasts. *Cell Signal* 2006 Dec; 18(12): 2131-42.
*These authors contributed equally to this work.

IV. **Tove Båge**, Johan Lindberg, Joakim Lundeberg, Thomas Modéer, Tülay Yucel-Lindberg. Signal pathways JNK and NF-κB, identified by global gene expression profiling, are involved in regulation of TNFα-induced mPGES-1 and COX-2 expression in gingival fibroblasts. *BMC Genomics.* 2010 Apr; 11: 241.

Contents

List of abbreviations

15d-PGJ_2	15-deoxy-Δ12,14-prostaglandin J_2
AA	arachidonic acid
Bay	Bay 11-7082
BIS	bisindolylmaleimide
BPB	4-bromophenacyl bromide
cDNA	complementary DNA
COX	cyclooxygenase
cPGES	cytosolic prostaglandin E synthase
$cPLA_2$	cytosolic phospholipase A_2
DE	differentially expressed
Dex	dexamethasone
DP	prostaglandin D_2 receptor
ECL	enhanced chemiluminescence
Egr-1	early growth response factor-1
EIA	enzyme immunoassay
EP	prostaglandin E_2 receptor
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FP	prostaglandin $F_{2\alpha}$ receptor
GCF	gingival crevicular fluid
GIVA PLA_2	group IV A phospholipase A_2
GO	Gene Ontology
GPRC	G-protein coupled receptor
GR	glucocorticoid receptor
HASM	human airway smooth muscle
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule-1
IkB	inhibitor of nuclear factor-κB
IL	interleukin
IL-1β	interleukin-1β
IP	prostacyclin receptor
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAP kinase	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MMP	matrix metalloproteinase
mPGES-1	microsomal prostaglandin E synthase-1
mPGES-2	membrane-associated prostaglandin E synthase-2
NF-κB	nuclear factor-κB
NSAID	non-steroidal anti-inflammatory drug
OPG	osteoprotegerin
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
PD	PD 153035 hydrochloride
PDTC	pyrrolidine dithiocarbamate
PGD_2	prostaglandin D_2
PGE synthase	prostaglandin E synthase
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
PKC	protein kinase C
PLA_2	phospholipase A_2
PMA	phorbol-12-myristate-13-acetate
PMN	polymorphonuclear leukocyte
PPAR-γ	peroxisome proliferator-activated receptor-γ
RANK	receptor activator of nuclear factor-κB
RANKL	receptor activator of nuclear factor-κB ligand
RIA	radioimmunoassay
RISC	RNA-induced silencer complex
Ro	Ro 106-9920
SB	SB 203580
siRNA	small interfering RNA
SP	SP600125
$sPLA_2$	secretory PLA_2
TNFα	tumour necrosis factor α
TP	thromboxane receptor
TXA_2	thromboxane A_2
VSMC	vascular smooth muscle cell

Introduction

The process of inflammation is vital for our survival. Without inflammation, wounds and infections would never heal and we would become helpless victims of our environment. However, inflammation can also work to our disadvantage. After an acute phase of inflammation in response to a bacterial infection, for example, the inflammation is generally resolved and healing can begin. When the inflammation is not resolved, we are instead left with an ongoing, chronic inflammation. In chronic inflammation, tissue destruction and healing are usually present at the same time, but the balance is delicate and can tilt towards destruction. Chronic inflammation is involved in many diseases, including rheumatoid arthritis, cardiovascular disease, cancer, type 2 diabetes mellitus and periodontitis. This work will focus on periodontitis, with special emphasis on prostaglandin E_2, but the elucidation of the molecular mechanisms behind the pathogenesis of periodontitis might one day lead to a new treatment strategy not only for periodontitis but also for other chronic inflammatory conditions.

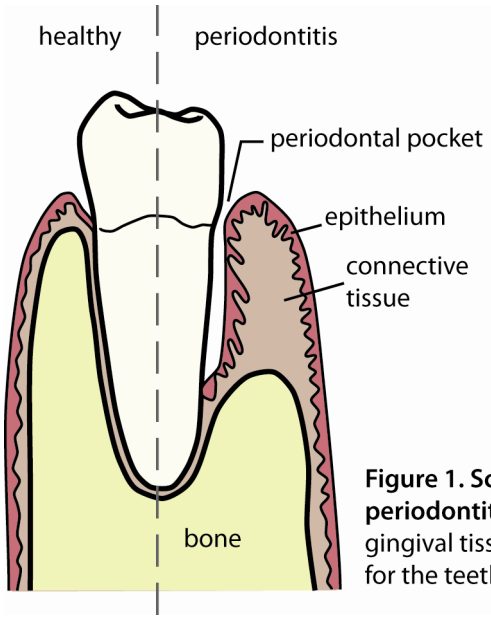

Figure 1. Schematic illustration of a healthy site and a periodontitis-affected site. In periodontitis, destruction of the gingival tissue and the alveolar bone results in reduced support for the teeth and may ultimately lead to tooth loss.

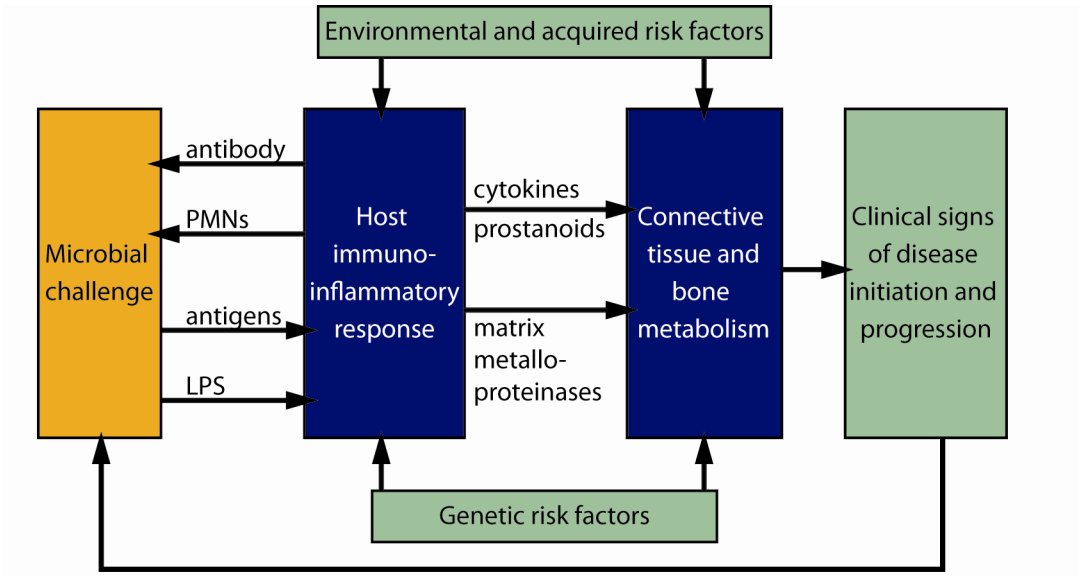

Figure 2. Conceptual model of periodontitis. The pathogenesis of periodontitis is a complex interaction between the microbial challenge, host immuno-inflammatory response and environmental and genetic risk factors. This illustration shows some of the components involved. Adapted from Page and Kornman 1997.[20]
Abbreviations: PMNs, polymorphonuclear leukocytes; LPS, lipopolysaccharide.

Pathogenesis of periodontitis

Periodontal disease, ranging from gingivitis to periodontitis, has probably been a factor of human life since prehistoric times.[1] Even Egyptian mummies from a thousand years BC have been shown to exhibit possible signs of periodontitis.[2] Today, periodontal disease is common and around 5-15% of the population suffer from severe periodontitis.[3,4] Periodontitis is not a life-threatening condition. Nevertheless, the loss of the bone supporting the tooth is irreversible and, furthermore, it has been suggested that there are associations between periodontal disease and systemic conditions such as heart disease, obesity and rheumatoid arthritis.[5-7]

Periodontitis is a chronic inflammatory state of the gingiva causing destruction of the gingival tissue, as well as the alveolar bone, resulting in reduced support for the teeth and ultimately tooth loss (Figure 1).[8-10] The definition of periodontitis is based on a number of clinical criteria, including bleeding on probing, pocket depth and clinical attachment loss.[11] However, the specific use of these criteria varies substantially between different studies.[11] The pathogenesis of periodontitis was gradually elucidated during the later half of the 20th century. In the 1960s and 1970s, research on humans and animals showed that bacteria play a critical role in initiating gingivitis and periodontitis.[12-14] Leading up to the 1980s, there were further advances within the field and the pivotal role of the host inflammatory response in disease progression began to emerge.[15-17] The importance of hereditary factors was subsequently demonstrated in several studies, including those comparing monozygotic and dizygotic twins.[18,19] Furthermore, systemic conditions and environmental factors such as smoking were also shown greatly to affect disease onset and progress.[20-22] Since over a decade, the concept of periodontitis pathogenesis has been considered to be a complex interaction between the microbial challenge, host response and environmental and genetic risk factors (Figure 2).[20] To summarise, bacterial components of the biofilm initiate an inflammatory cascade, including the infiltration of immune cells and the production of

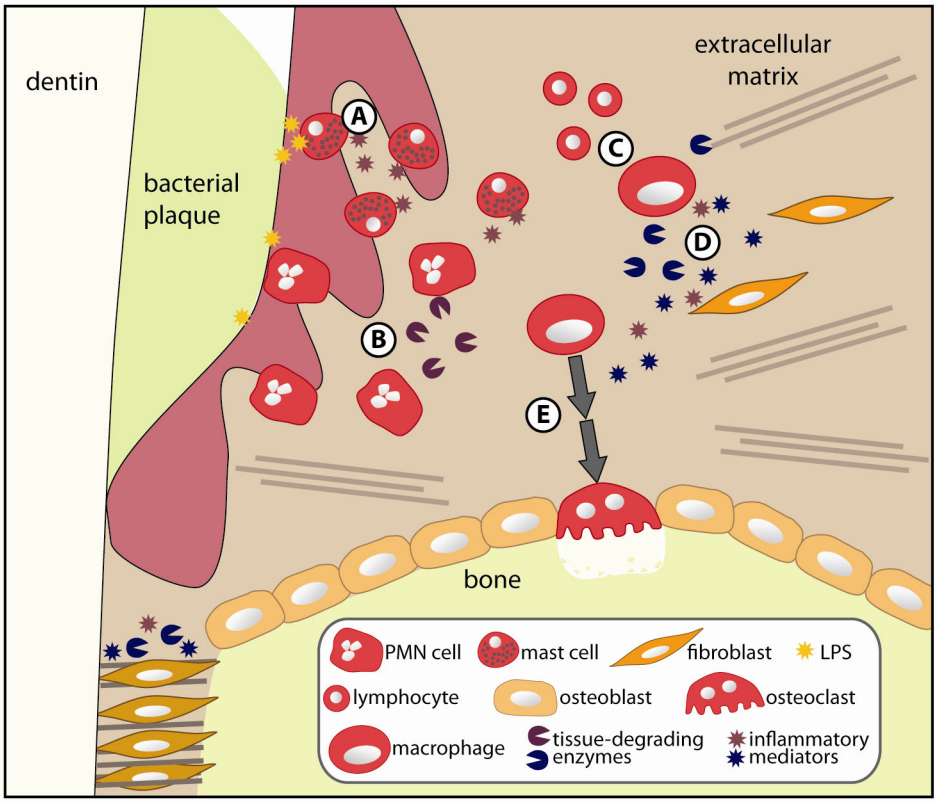

Figure 3. Host response and bone degradation in periodontitis. The host response in periodontitis includes complex interactions between a multitude of cell types and inflammatory mediators, some of which are illustrated above. **A)** Bacterial antigens, such as lipopolysaccharide (LPS), stimulate mast cells to release vasoactive amines and pre-formed tumour necrosis factor α. **B)** Polymorphonuclear (PMN) leukocytes are recruited into the tissue and release lysosomal enzymes. **C)** Lymphocytes and macrophages invade the tissue. **D)** Inflammatory cells and resident cells produce inflammatory mediators (cytokines and prostaglandins), as well as proteolytic enzymes. **E)** Macrophages form pre-osteoclasts which then mature into osteoclasts, capable of degrading the alveolar bone. Adapted from Lerner 2005.[31]

inflammatory mediators in the periodontal tissue, which results in soft tissue disintegration and bone destruction. This process will be discussed in more detail in the following sections.

Bacterial challenge

Bacterial components, such as lipopolysaccharides (LPS), peptidoglycans and lipotechoic acids, that instigate the inflammatory reaction can be found in the biofilm on the tooth surface.[21,22] The biofilm starts to form when bacteria adhere to the surface of salivary proteins naturally covering the tooth.[23] The presence of bacteria on the surface then facilitates the adhesion of other bacteria and an extracellular matrix containing complex carbohydrates is eventually secreted.[23] This matrix makes the biofilm more resistant to physical removal, by toothbrushing, for example.[23] The different bacteria of the biofilm can compete, using antimicrobial peptides, but they can also form symbiotic relationships that promote mutualistic growth.[23] The general presence of bacteria in the oral cavity is not harmful *per se* and a microflora containing several hundred species can be found in dental plaque without the presence of periodontitis. As periodontitis develops, however, the composition of the microflora shifts away from beneficial species and towards species associated with periodontal disease.[24] The current literature points to particular pathogens

commonly associated with disease, including *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*.[25,26]

The physical removal of bacterial plaque and calculus is currently the main approach to treating periodontitis. Together with root planing and scaling, locally or systemically administered antibiotics have also been suggested to have a beneficial effect on treatment outcome.[27,28] A different approach, which is still not feasible for common practice but is possible in principle, is to utilise a vaccine against specific detrimental bacterial species such as *P. gingivalis*.[29] It has also been suggested that beneficial bacteria might be administered as probiotics to shift the balance towards a healthy microflora as a treatment for periodontitis.[30] However, treatment strategies for periodontitis need not be limited to bacterial intervention. An in-depth understanding of the host response could potentially reveal targets for additional therapies that may ameliorate disease progress.

Host immuno-inflammatory response

The host response to the bacterial challenge includes the action and stimulation of different inflammatory cell types, as well as resident cells of the tissue (Figure 3).[20,31-33] Bacterial antigens, such as LPS and peptidoglycans, are recognised by toll-like receptors on the surface of host cells, which initiates an inflammatory response.[34] Through a cascade of events, mast cells are stimulated to release vasoactive amines and pre-formed tumour necrosis factor α (TNFα), contributing to increased vascular permeability, as well as the increased expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and P-selectin, on endothelial cell surfaces.[21,33] This process recruits polymorphonuclear leukocytes (PMNs) into the tissue, which then release lysosomal enzymes, contributing to tissue degradation.[33] Lymphocytes, mainly including T-cells, and macrophages further invade the tissue. At this point, 60-70% of the collagen in the gingival connective tissue is degraded at the site of the lesion, but the bone is still intact.[15,33] At this stage, it is still possible for the gingival tissues to repair and remodel without permanent damage. However, the inflammation may change character to a B-cell/plasma cell response, with subsequent connective tissue breakdown and irreversible bone loss.[33] In this scenario, macrophages form pre-osteoclasts which then mature into osteoclasts, capable of degrading the alveolar bone.[35]

In addition to the invading inflammatory cells, which produce inflammatory mediators and drive the process of inflammation, the resident cells of the gingival tissue may also affect the progression of periodontitis. The vessels, consisting of endothelial cells and smooth muscle cells, are the first to come in contact with circulatory inflammatory cells invading the tissue. In response to TNFα, endothelial cells express the surface adhesion molecules which attract the inflammatory cells to the site of inflammation.[21,33] In gingival connective tissue, the most ubiquitous resident cells are gingival fibroblasts. By producing inflammatory mediators, such as cytokines, chemokines, proteolytic enzymes and prostaglandins, these cells participate in the inflammatory response and contribute to disease persistence.[36-40] The periodontal ligament fibroblasts that are located between the tooth and the alveolar bone are also involved in the inflammatory reaction, producing inflammatory mediators, such as prostaglandins, proteolytic enzymes and factors affecting bone resorption.[41-43]

Inflammatory mediators

Throughout each step of the process of inflammation, pro-inflammatory mediators are released and affect different cell types to propel the inflammatory cascade. These mediators include pro-inflammatory cytokines and chemokines, proteolytic enzymes and prostaglandins.

Several pro-inflammatory cytokines are involved in the pathogenesis of periodontitis, including the prominent TNFα, interleukin-1 (IL-1) and interleukin-6 (IL-6). The cytokines IL-1 and IL-6 are abundantly produced in the B-cell/plasma cell response which characterises the progression of periodontitis.[33] IL-6 is produced by epithelial cells, lymphocytes, monocytes and fibroblasts in response to bacterial LPS, IL-1 and TNFα and has been shown to stimulate the formation of osteoclasts *in vitro*.[21,44] Increased levels of IL-6 have been demonstrated in the gingival crevicular fluid (GCF) of patients with periodontitis, compared with healthy controls, and higher expression of IL-6 was reported in diseased gingival tissues when compared with healthy ones in periodontitis patients.[45,46]

The inflammatory cytokines IL-1 and TNFα play an especially central role in the pathogenesis of periodontitis[47] and are used in our *in vitro* model of inflammation throughout this thesis. As mentioned above, TNFα is involved from early on in the inflammatory cascade, as it is released from mast cells in response to bacterial challenge. In the clinical context, TNFα and IL-1β have been found in increased concentrations in the GCF and gingival tissue of periodontitis sites.[48-50] The pivotal role of these cytokines in periodontitis is further supported by reports that attachment loss is reduced in periodontitis patients with rheumatoid arthritis after anti-TNF treatment and that the administration of recombinant TNFα or IL-1 to the gingiva has been shown to exacerbate experimental periodontitis in rats.[51-53] In addition, soluble receptors of IL-1 and TNF have been shown greatly to inhibit the progress of periodontitis in a primate model.[54,55] At cellular level, these two cytokines are involved in the induction of several other inflammatory mediators, such as IL-6, IL-8, matrix metalloproteinases (MMPs) and prostaglandin E_2.[8,21,56-58] TNFα and IL-1 are produced by many cell types in periodontal tissue: monocytes/macrophages, PMN cells, fibroblasts, epithelial cells, endothelial cells and osteoblasts.[47] To summarise, these two cytokines occupy a spider-in-the-web position among mediators of the inflammatory cascade in periodontitis.

Chemokines are cytokines involved in inducing chemotaxis in responsive cells. The chemokines IL-8 and monocyte chemoattractant protein-1 (MCP-1) attract neutrophils and other leukocytes to the site of inflammation. IL-8 is secreted by many different cells, including monocytes, lymphocytes, epithelial cells, endothelial cells and fibroblasts, in response to IL-1, TNFα and LPS.[8,59] High levels of IL-8 expression have been shown to be localised to sites with high concentrations of PMN cells in gingival tissue from patients with periodontitis.[60] Furthermore, increased levels of IL-8 have been demonstrated in GCF from periodontitis sites compared to healthy control sites and the levels of IL-8 decreased after periodontal therapy.[61] The chemokine MCP-1 is produced by endothelial cells, epithelial cells and fibroblasts in response to bacterial components, such as LPS, or inflammatory mediators.[21,62] The involvement of MCP-1 in periodontitis is supported by studies demonstrating increased levels of the chemokine in gingival biopsies and GCF from patients with periodontitis, as well as decreased MCP-1 in GCF after the treatment of periodontitis.[49,63,64]

The proteolytic MMP enzymes and their endogenous inhibitors, termed tissue inhibitors of metalloproteinases, are involved in the homeostasis of the extracellular matrix in healthy tissue, but they are also key players in the process of inflammation and tissue degradation. In periodontitis, TNFα and IL-1β stimulate fibroblasts to secrete an excess of MMPs that degrade the extracellular matrix.[21] MMP-8, one of the several MMPs involved in periodontitis, has been observed in increased levels in periodontitis, as well as in subsequently reduced levels after treatment.[65]

Among prostaglandins, prostaglandin E_2 (PGE_2) is the most prominent in the pathogenesis of periodontitis.[66,67] PGE_2 is produced by immune cells as well as fibroblasts and other resident cells of the gingival tissue, and has a wide range of biological effects on the cells of the diseased gingiva.[36,66] The effects of PGE_2 include the stimulation of inflammatory mediators and MMPs, as well as osteoclast formation through the receptor activator of nuclear factor-κB ligand (RANKL).[66,68] Prostaglandins, especially PGE_2, are central to the work in this thesis and will therefore be discussed in detail in the following chapters.

The multitude of inflammatory processes involved in the host response provides an equal multitude of possible targets for therapeutic intervention. Modulation of the host response is a possible treatment strategy for periodontitis, in conjunction with the physical removal of the microbial challenge. Patients with periodontitis who are treated with non-steroidal anti-inflammatory drugs (NSAIDs) have shown reduced periodontal disease progression and reduced alveolar bone resorption.[69,70] However, a cross-sectional study by Heasman and Seymour[71] demonstrated no differences in alveolar bone loss or other clinical parameters when comparing patients on long-term NSAID therapy with controls not treated with NSAIDs. It has also been shown that the antibacterial substance triclosan, which is used in toothpastes, has anti-inflammatory effects and enhances the healing response in chronic periodontitis patients.[72-74] Interestingly, triclosan has been shown to reduce the levels of PGE_2, suggesting that the anti-inflammatory effect of triclosan may be partly mediated by decreased PGE_2 levels.[75,76] The following chapters in this thesis are dedicated to prostaglandins and to PGE_2 in particular.

Prostaglandins

The Nobel Prize in Physiology or Medicine in 1982 was awarded jointly to Sune K. Bergström, Bengt I. Samuelsson and John R. Vane "for their discoveries concerning prostaglandins and related biologically active substances". However, the story of prostaglandins began much earlier. About a century ago, it was discovered that the urinary bladder and blood pressure of dogs was affected by an extract from the human prostate gland.[77] In 1930, Kurzrok and Lieb showed that human semen caused contraction of the uterus.[78] These and other findings subsequently led to the discovery of substances in human seminal plasma that had substantial biological effects. The name "prostaglandin" refers to the origin of the substances, which was seemingly the prostate gland.[79] In 1960, the Swedish researchers Sune K. Bergström and Jan Sjövall published their isolation of two separate substances from sheep seminal vesicles.[80,81] These two compounds were named after the respective solvent in which they were soluble; prostaglandin E for ether and prostaglandin F for phosphate buffer, *fosfatbuffert* in Swedish. The structures of these prostaglandins were subsequently elucidated by Bergström and his graduate student Bengt I. Samuelsson.[82]

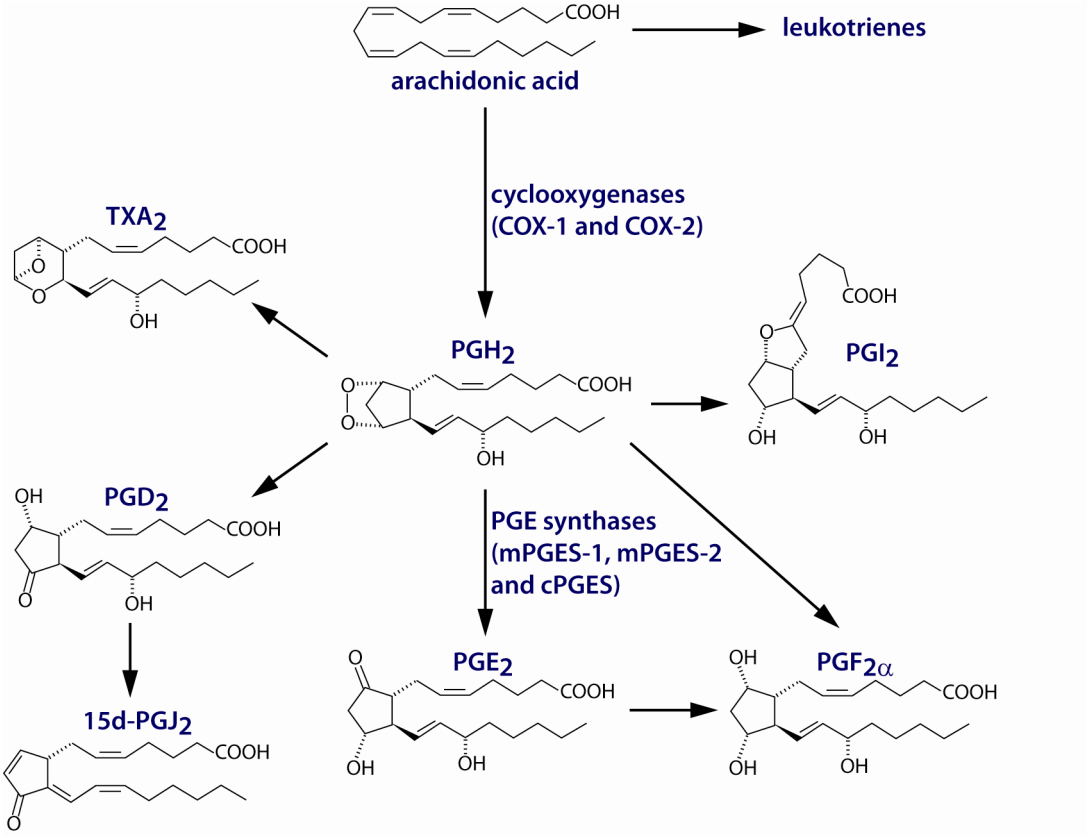

Figure 4. Overview of prostanoid biosynthesis. Arachidonic acid, derived from membrane lipids, can be metabolised by a range of enzymes to form prostaglandins (including PGD_2, PGE_2, 15d-PGJ_2 and $PGF_{2\alpha}$), prostacyclin (PGI_2) and thromboxanes (including TXA_2), as well as leukotrienes.

Abbreviations: 15d-PGJ_2, 15-deoxy-Δ12,14-prostaglandin J2; COX, cyclooxygenase; cPGES, cytosolic PGE synthase; mPGES-1, microsomal PGE synthase-1; mPGES-2, membrane-associated PGE synthase-2; PGE synthases, prostaglandin E synthases; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; TXA_2, thromboxane A_2.

Today, several different prostaglandins have been characterised. Almost all organs can be affected by prostaglandins and they can be produced by most of the cells of the human body. Their action is autocrine or paracrine, which means that the signalling of prostaglandins is localised to the immediate surroundings of the site of synthesis. They are not stored in the cell but are synthesised *de novo* as a result of cell activation by cytokines, growth factors or other stimuli. The biosynthesis of all prostanoids (prostaglandins, prostacyclins and thromboxanes) begins with the conversion of membrane phospholipids to arachidonic acid (AA), which can then be further metabolised by a range of enzymes to form different prostaglandins. AA can also be converted into leukotrienes, which are produced mainly in inflammatory cells and are involved in allergic reactions, as well as in sustaining inflammatory reactions.[83,84] An overview of prostanoid biosynthesis is given in Figure 4.

Prostaglandins are secreted from the cell through the plasma membrane by a prostaglandin transporter protein.[85] The actions of prostaglandins on target cells are then dependent on the prostaglandin receptors on their surfaces. Currently, nine prostaglandin receptors have been characterised, not counting splice variants that differ only slightly from one another.[86] These receptors include the four receptors EP_1-EP_4 for PGE_2, DP_1 and DP_2 for PGD_2, as well as IP, FP and TP for PGI_2, $PGF_{2\alpha}$ and TXA_2 respectively.[84] The prostaglandin receptors are G-protein-coupled receptors (GPCRs), apart from DP_2, which

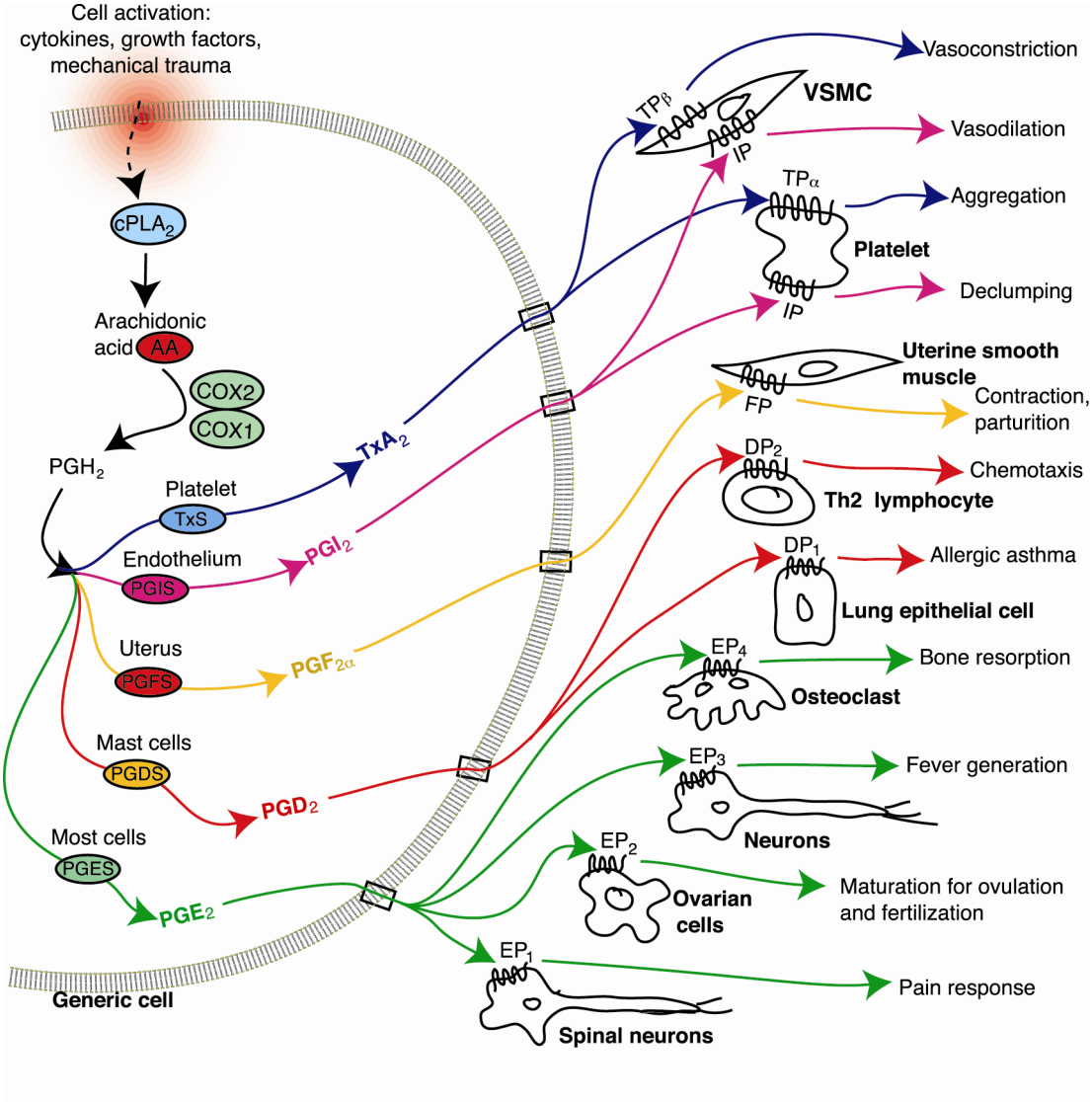

Figure 5. Prostanoid synthesis and examples of the actions of prostanoids on target cells. In response to stimuli, prostanoids are synthesised and secreted. The prostanoids then affect cells by binding to their respective receptors on the cell surfaces. Adapted from Funk 2001.[84]

Abbreviations: COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; DP, PGD_2 receptor; EP, PGE_2 receptor; FP, $PGF_{2\alpha}$ receptor; IP, PGI_2 receptor; PGD_2, prostaglandin D_2, PGDS, PGD synthase; PGE_2, prostaglandin E_2; PGES, PGE synthase; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGFS, PGF synthase; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; PGIS, PGI synthase; TP, TXA_2 receptor; TXA_2, thromboxane A_2; TXS, TXA synthase; VSMC, vascular smooth muscle cell.

is grouped with the chemoattractant receptors. Most of the prostaglandin GPCRs are found in the plasma membrane, but receptors can also be localised to the nuclear envelope.[87,88] Examples of the many diverse actions of different prostaglandins on target cells throughout the human body are illustrated in Figure 5 which is adapted from Funk 2001.[84] The single prostaglandin that has merited the largest number of research publications to date is PGE_2. It has been implicated in such diverse processes as rheumatoid arthritis,[89-91] periodontitis,[92,93] atherosclerosis,[94,95] embryonic development,[96] hyperalgesia,[97] chronic constipation[98] and skin tumour progression.[99] Regarding periodontitis, concentrations of PGE_2 are enhanced in periodontal tissue and GCF of periodontitis patients, compared with periodontally healthy controls.[92,93] Moreover, the enhanced levels of PGE_2 in periodontitis correlate well with disease severity as measured by attachment loss.[100]

Prostaglandin E_2

Prostaglandin E_2 is involved in many different biological processes throughout the human body. *In vivo*, PGE_2 has a half-life of less than one minute.[101] It is generally degraded in several steps to dicarboxylic acids, which are excreted into the urine, but can also be reduced by PGE 9-ketoreductase to form $PGF_{2\alpha}$.[102,103] The short *in vivo* half-life of PGE_2 is consistent with its autocrine or paracrine action on the cells of the tissue. The effect of PGE_2 on a specific cell type depends on the prostaglandin receptors, EP_1 through EP_4. The receptors EP_1 and EP_3 are expressed by neurons, among other cells, and cause fever and pain response; EP_1 through enhanced intracellular Ca^{2+} levels and EP_3 through the inhibition of adenylate cyclase.[84,104] EP_2 and EP_4, which are reported to activate adenylate cyclase and protein kinase A signalling, are more relevant to the pathogenesis of periodontitis.[105] In rodent models, these two receptors have been shown to be involved in bone resorption in response to PGE_2.[106,107] The major pathway by which PGE_2 stimulates bone resorption is generally considered to be via the up-regulation of RANKL expression and the inhibition of osteoprotegerin (OPG) expression in osteoblastic cells.[105] OPG acts as a decoy receptor for RANKL and the inhibition of its expression enables RANKL to interact with its receptor RANK on other cells. RANKL then binds to RANK on osteoclast lineage cells to drive differentiation to osteoclasts,[35,105] as schematically illustrated in Figure 6 (adapted from Blackwell et al. 2010[105]). In osteoclastogenesis, the stimulatory effect of oral pathogen sonicates has been demonstrated to be primarily mediated through the PGE_2/RANKL pathway in primary mouse osteoblasts co-cultured with bone marrow cells.[108] It has also been reported that RANKL-stimulated osteoclastogenesis can be enhanced by PGE_2 and LPS through direct effects on the hematopoietic cell lineage.[68] PGE_2 has been shown both to inhibit and stimulate OPG expression,[43,109] a contradiction which may be the result of differing incubation times, as has been suggested for the effect of PGE_2 on osteoclast formation.[105] Inflammatory cytokines such as IL-1β induce RANKL and/or OPG expression in several cell types, including osteoblasts, gingival fibroblasts and periodontal ligament fibroblasts.[43,110]

In addition to its effect on bone resorption, PGE_2 can also stimulate bone formation in cell cultures, as well as *in vivo*.[105,111-113] Interestingly, intermittent PGE_2 administration has been shown to lead to bone formation in rats, whereas continuous administration promotes bone resorption.[114] However, the mechanisms behind the balance between bone formation and resorption with regard to PGE_2 are still largely unresolved and may depend on the cellular environment.[105]

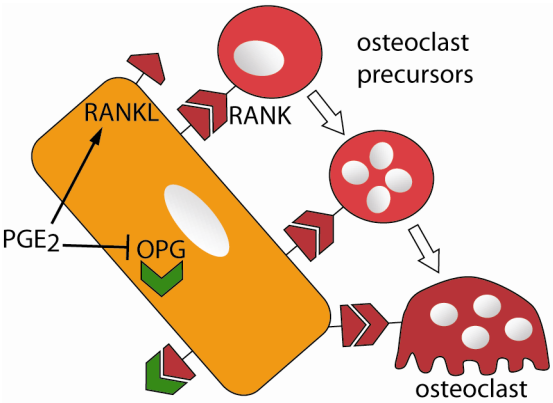

Figure 6. Potential stimulation of osteoclast formation by PGE_2 via RANKL. PGE_2 can increase the expression of RANKL and decrease the expression of its decoy receptor OPG in osteoblastic cells. The binding of RANKL to RANK on osteoclast precursors then stimulates the formation of osteoclasts. Adapted from Blackwell et al. 2010.[105]
Abbreviations: OPG, osteoprotegerin; PGE_2, prostaglandin E_2; RANK, receptor activator of NF-κB; RANKL, RANK ligand.

Levels of PGE_2 can be modulated by regulation of the enzymes involved in its synthesis. In the light of this, it is essential to understand the biosynthetic pathway of PGE_2, as well as the intracellular signalling pathways that regulate its enzymes.

Prostaglandin E_2 biosynthesis

The biosynthesis of PGE_2 involves three different groups of enzymes acting sequentially (see Figure 4, page 8). The first group of enzymes, phospholipase A_2 (PLA_2), converts membrane lipids to AA.[115,116] The second group of isoenzymes is the cyclooxygenases (COX), which convert AA to prostaglandin H_2 (PGH_2).[117] The intermediate prostaglandin PGH_2 is in turn metabolised to diverse prostaglandins, including PGE_2, PGF_2, PGD_2, and PGI_2, by multiple enzymes.[117,118] The third group of isoenzymes, prostaglandin E synthase (PGE synthase), which is the terminal enzyme in the synthesis of PGE_2, catalyses the conversion of COX-derived PGH_2 to PGE_2.[119,120]

Phospholipase A_2

The first step of PGE_2 synthesis is the conversion of membrane lipids into AA, a reaction which is catalysed by the PLA_2 enzymes. Of the more than 20 currently identified mammalian intracellular PLA_2 isoforms, $cPLA_2\alpha$ (systematically termed Group IV A PLA_2 or GIVA PLA_2 for short) is the main player in AA release.[121] The first GIVA PLA_2 was identified in 1986 in human platelets[122] and was cloned and sequenced in 1991.[123] The $cPLA_2\alpha$ enzyme is recruited to intracellular membranes by a Ca^{2+}-dependent process.[124] It can be activated by many different signals, including phosphorylation and the second messenger phosphatidylinositol.[124] The expression of $cPLA_2$(alpha?) can be stimulated by TNFα or IL-1β, as demonstrated in several cell types, including gingival fibroblasts and rheumatoid synovial fibroblasts.[125,126] Insights into the role of $cPLA_2\alpha$ can be obtained from studies of knockout mice, demonstrating that the effects of many inflammatory conditions are reduced, including damage from acute lung injury, collagen-induced arthritis and inflammatory bone resorption.[127-129] High levels of PLA_2 activity in the GCF of patients with periodontitis have been shown to decrease after periodontal treatment.[130] Moreover, secretory PLA_2, $sPLA_2$, is present in increased amounts in the synovial fluids and sera of patients with rheumatoid arthritis,[131,132] further emphasising the role of PLA_2 enzymes in inflammatory conditions. Several $cPLA_2\alpha$ inhibitors have been developed and beneficial effects have been observed in models of inflammatory conditions including rheumatoid arthritis and osteoarthritis.[133] Pharmaceuticals such as darapladib and varespladib, which inhibit lipoprotein-associated PLA_2 and $sPLA_2$, respectively, are currently undergoing clinical studies for use in atherosclerosis.[134,135] However, inhibition of PLA_2 affects the synthesis of all prostanoids and leukotrienes, which may result in unwanted side-effects. Bearing this in mind, we now move further down the cascade of PGE_2 synthesis.

Cyclooxygenase

The cyclooxygenases converting PLA_2-derived AA to PGH_2 have currently been described in two different isoforms, COX-1[136] and COX-2.[137,138] COX-1 is mainly involved in immediate prostaglandin production, initiated by agonists that cause a rapid burst of AA by affecting intracellular Ca^{2+}.[124] It is constitutively expressed in most tissues and is involved in maintaining tissue homeostasis. COX-1 expression has been shown to be unaffected by inflammatory stimuli in several cell types *in vitro*, including gingival

fibroblasts.[58,139-141] The second isoform, COX-2, is essential for delayed prostaglandin production, where AA, induced by pro-inflammatory stimuli, is gradually released over longer time periods.[142,143] However, COX-2 can also be involved in immediate prostaglandin synthesis if its expression has already been increased through inflammatory stimuli.[143] The importance of the COX-2 isoform in inflammation is further indicated by its observed up-regulation in several chronic inflammatory conditions such as rheumatoid arthritis, Crohn's disease and periodontitis.[144-146]

The COX enzymes are located in the endoplasmic reticulum (ER) and nuclear envelope, with COX-2 more concentrated in the nuclear envelope.[147] COX-1 and COX-2 are 60% identical in amino acid sequence and their three-dimensional structures are almost superimposable. A splice variant of the COX-1 gene, retaining an intron which is not included in COX-1 mRNA, has been putatively termed COX-3 and suggested as a possible target for acetaminophen in canine systems.[148] However, the relevance of these data to the human context has been questioned and, since the inclusion of the intron causes a shift in the reading frame resulting in a severely truncated enzyme with no COX activity, the importance of the so-called COX-3 enzyme appears to be very limited.[149]

The COX-1 and COX-2 enzymes convert AA to PGH_2 in two steps, each catalysed by a separate active site of the enzyme.[150] First, the cyclooxygenase active site catalyses the formation of the intermediate PGG_2 from AA. Then, at the peroxidase active site of the COX enzyme, PGG_2 is reduced to PGH_2. As first suggested by Nobel laureate John R. Vane in 1971,[151] the cyclooxygenases are the primary targets for NSAIDs such as aspirin. NSAIDs inhibit the first step of the reaction, the formation of PGG_2. Specific COX-2 inhibitors have been developed to achieve inhibition of inflammation-induced PGE_2 production without the detrimental inhibition of baseline, COX-1-derived prostaglandin production thought to account for the gastrointestinal side-effects of traditional NSAIDs.[150] However, it has been shown that these drugs also have several side-effects, including cardiovascular problems,[152] and one of the COX-2-specific pharmaceutical inhibitors, Vioxx, was withdrawn from the market due to these side-effects. The problems experienced when inhibiting the COX enzymes draw our attention even further down the cascade of PGE_2 synthesis.

The terminal step in PGE_2 biosynthesis is catalysed by the PGE synthases. Currently, three different PGE synthases have been identified and cloned; the inducible, microsomal and glutathione-dependent PGE synthase, mPGES-1,[119,153] the constitutively and widely expressed cytosolic PGE synthase, cPGES,[154] and the later characterised membrane-associated and glutathione-independent PGE synthase, mPGES-2.[155]

Microsomal prostaglandin E synthase-1 (mPGES-1)

The identification of mPGES-1 was reported in 1999 by Per-Johan Jakobsson et al. at Karolinska Institutet.[119] The expression of mPGES-1 under normal conditions is low in most tissues,[119] but it increases considerably after inflammatory stimuli such as TNFα, IL-1β or LPS, leading to increased PGE_2 synthesis.[76,156-161] The involvement of mPGES-1 in inflammatory conditions such as rheumatoid arthritis[162,163] and gastritis,[158] as well as in carcinogenesis[164,165] has been demonstrated, but limited information is available regarding the role of mPGES-1 in periodontitis. *In vitro*, the induction of mPGES-1 in response to IL-1β and TNFα has been reported in various cell types, including cultured gastric fibroblasts, synovial fibroblasts, cardiac fibroblasts, gastric cancer cell lines, monocytes and

gingival fibroblasts.[76,157-161,166,167] Furthermore, studies have suggested that mPGES-1 displays functional coupling with COX-2 to promote delayed PGE_2 synthesis.[168,169] This suggestion is supported by the findings that mPGES-1 and COX-2 are co-induced by inflammatory stimuli in mouse macrophages and osteoblasts, fibroblasts, chondrocytes and cancer cells.[170-172] However, the absoluteness of this coupling has been questioned and a lack of exclusive co-ordination between these two enzymes has been reported.[173-175]

The mPGES-1 enzyme is a 16 kDa membrane-spanning protein, which requires the co-factor glutathione to function.[119] It is located in the ER and the perinuclear area and appears to be co-localised with COX-2,[143,168] possibly facilitating the concerted action of these enzymes in inflammation-induced PGE_2 synthesis. The role of mPGES-1 in inflammation-induced PGE_2 has been studied using antisense oligonucleotides blocking mPGES-1, as well as mPGES-1 knockout mice.[176-178] In most respects, the knockout mice are indistinguishable from their wild-type littermates, apart from a marked difference in inflammatory reactions in disease models, such as collagen-induced arthritis and inflammatory pain.[179] For instance, the effects of collagen-induced arthritis were greatly reduced in the knockout mice.[179] Furthermore, in mPGES-1-null mice, no augmentation of PGE_2 production was observed in response to LPS, indicating that mPGES-1 is essential for LPS-induced PGE_2 production.[176,178] In addition, blocking mPGES-1 expression using an antisense oligonucleotide inhibited osteoclastogenesis and bone resorption in mouse osteoblasts stimulated by the cytokines IL-1β and TNFα.[170] Taken together, these findings indicate that mPGES-1 plays a crucial role in inflammation-induced PGE_2 production and thereby also possibly in the pathogenesis of periodontitis. The endogenous and non-endogenous regulation of mPGES-1 expression and activity will be discussed in detail in the next chapter.

Membrane-associated prostaglandin E synthase-2 (mPGES-2)

A glutathione-independent, membrane-bound PGE synthase isoform was observed in rat organs in 1997[120] and this PGE synthase isoform, mPGES-2, was finally cloned and characterised in 2002.[155] It is a homodimeric protein, where the molecular weight of each monomer is 33 kDa.[155] It is synthesised as a Golgi membrane-associated protein, but the proteolytic removal of a hydrophobic N-terminal domain results in a mature cytosolic enzyme.[143] In contrast to mPGES-1, the enzyme mPGES-2 appears to exhibit no selective coupling with COX-1 or COX-2.[143] The mPGES-2 enzyme has not been as extensively studied as mPGES-1 and the expression and inducibility vary between different cell types, in response to different inflammatory stimuli.[120,158,180,181] Knockout mice lacking the mPGES-2 gene display no decrease in PGE_2 levels, indicating that this enzyme is not essential for *in vivo* PGE_2 synthesis.[182] However, a study by Kubota et al.[183] of mPGES-1 knockout mice indicated that mPGES-2 may play a compensatory role in inflammation-induced PGE_2 production in myometrium, substituting for mPGES-1. On the other hand, mPGES-2 expression was not increased by LPS treatment in microglia derived from mPGES-1 knockout mice in a study by Ikeda-Matsuo et al.,[177] further highlighting the different roles of mPGES-2 in different tissues.

Cytosolic prostaglandin E synthase (cPGES)

The cytosolic PGE synthase was discovered in 1994[184] as a conserved protein of broad tissue distribution with a molecular weight of 23 kDa, but its PGE synthase activity was first characterised in 2000.[154] The enzyme cPGES is considered constitutively expressed, as

shown in various tissues, as well as in cultured cells such as gastric fibroblasts and rheumatoid synovicytes.[154,158,185] Based on co-transfection studies, as well as the co-localisation of cPGES and COX-1, these enzymes are considered to be functionally coupled.[154,186] However, there have been reports of increased cPGES expression after LPS or IL-1β stimulation of rodent brain.[154,187] Knockout mice lacking the cPGES gene do not survive the perinatal period.[188] Although cPGES appears to be uninvolved in inflammatory-increased PGE_2 synthesis in most tissues, a recent study suggests that cPGES might be involved in regulating the degradation of PGE_2 in mouse embryonic fibroblasts and rat fibroblasts.[189]

Regulation of mPGES-1

Although the regulation of COX-2 and PGE_2 production has been intensively investigated in various cell types, the signal transduction pathways regulating the expression of mPGES-1 in relation to PGE_2 have not been adequately explored. Various intracellular signalling pathways have been reported to be involved in inflammation-induced PGE_2 production and in the expression of PGE_2-sythesising enzymes, although these pathways appear to be both cell and stimulus specific. Protein kinase C (PKC) and mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinase (ERK) and p38 MAP kinase, have been reported to be involved in inducing mPGES-1 expression in IL-1β-stimulated colonocytes and orbital fibroblasts, as well as in rat aortic adventitial fibroblasts.[190-192] The ERK and p38 MAPK pathways were also shown to be involved in IL-1β-stimulated mPGES-1 expression in human chondrocytes from patients with osteoarthritis.[193] However, mPGES-1 expression has been shown to be unaffected by PKC stimulation in gastric carcinoma cell lines[161] and by the inhibition of p38 MAPK in cardiac fibroblasts,[160] which exemplifies the cell-specific nature of mPGES-1 regulation. In rat microglia, LPS-induced mPGES-1 expression was shown to be mediated by the phosphatidylinositol 3-kinase/Akt, PKC, c-Jun N-terminal kinase (JNK), and nuclear factor-κB (NF-κB) signalling pathways.[194] The JNK pathway has also been shown to be involved in increased mPGES-1 expression in IL-1β-stimulated cells, such as cardiac fibroblasts and A549 human lung epithelial cells.[160,195]

The mPGES-1 promoter contains regulatory elements including activator protein-1, glucocorticoid receptor (GR) and early growth response-1 (Egr-1) binding sites.[196,197] The transcription factors Egr-1 and NF-κB have been shown to be involved in the regulation of mPGES-1 expression in different cell types including HeLa cells, pulmonary A549 cells, murine osteoblastic cells and murine macrophages.[172,198-200] In gingival fibroblasts, we have previously reported that the inflammatory mediators IL-1β and TNFα induce the expression of mPGES-1 in parallel with PGE_2 production and that the glucocorticoid dexamethasone inhibits the expression of mPGES-1.[157]

In addition to elucidating the endogenous regulation of the PGE synthases, great efforts have been made to develop a strategy for non-endogenous regulation of mPGES-1. Recently, several different groups of compounds inhibiting mPGES-1 activity have been described.[201,202] One of the most promising groups of inhibitors are the disubstituted phenantrene imidazoles, which were also orally active in a guinea pig model.[203] The indole 5-lipoxygenase-activating protein inhibitor MK-886 and its derivatives have also been shown to inhibit mPGES-1 in enzyme assays and, less potently, inhibit PGE_2 formation in cell-based assays.[171,204,205] Furthermore, natural products such as curcumin[206] (from the

spice turmeric) and epigallocatechin-3-gallate[207] (from green tea) have been shown to affect mPGES-1 *in vitro*. Several mPGES-1 inhibitors are being tried out in animal models, but none are as yet available for use in humans.[208-211]

To summarise the central points of this introduction, the product of PGE synthase, PGE_2, is widely recognised as one of the key inflammatory mediators in the pathogenesis of periodontitis. The expression of PGE synthases has been studied in several diseases, including rheumatoid arthritis, colon cancer and gastritis. However, there are no previously published data on the expression of the three PGE synthases in periodontitis. Furthermore, there are no reports addressing the contribution of the different cells in the connective tissue to inflammation-induced PGE_2 production. The signal pathways leading to increased mPGES-1 expression in response to inflammatory stimuli may represent targets for the possible interruption of the increase in PGE_2 production in inflammation, which makes it important to study the intracellular pathways in a cell type relevant to the context of periodontitis.

Aims of the thesis

General aim

The general aim of this thesis was to investigate the expression and regulation of the PGE synthases *in vivo* in periodontitis-affected gingival tissue and *in vitro* in cell cultures.

Specific aims

Study I

The aim of Study I was to investigate the localisation and expression of PGE_2-producing enzymes, focusing on the PGE synthases (mPGES-1, mPGES-2 and cPGES), in inflamed human gingival connective tissue collected from patients with periodontitis. An additional aim was to investigate the regulation of these enzymes using *in vitro* models, mimicking an inflammatory situation.

Study II

The aim of Study II was to elucidate the impact of down-regulated mPGES-1 expression on the expression of the mPGES-2 and cPGES isoenzymes, as well as on PGE_2 production, using small interfering RNA (siRNA) technology and chemical inhibition.

Study III

The aim of Study III was to investigate the intracellular regulation of mPGES-1 expression with special regard to PLA_2, PKC, tyrosine kinases, p38 MAP kinase, prostaglandin metabolites and the transcription factors Egr-1, GR and NF-κB in gingival fibroblasts stimulated with the inflammatory cytokines TNFα and IL-1β.

Study IV

The aim of Study IV was to further explore the signal transduction pathways involved in the regulation of mPGES-1 and COX-2 in TNFα-stimulated primary gingival fibroblasts through a global gene expression approach, using microarray technology.

Materials and methods

This section gives a brief overview of the methods used to obtain the results presented in this thesis. The methods mainly focus on gene expression and the regulation of gene expression, focusing on the PGE synthases (Figure 7). For more detailed protocols, including in-depth details such as concentrations of substances and buffers, primer sequences or sources of chemicals, the reader is referred to the materials and methods sections of the published studies (I-IV). To facilitate the understanding of Studies I-IV by researchers unfamiliar with molecular methods, the principles underlying the methods are also briefly covered.

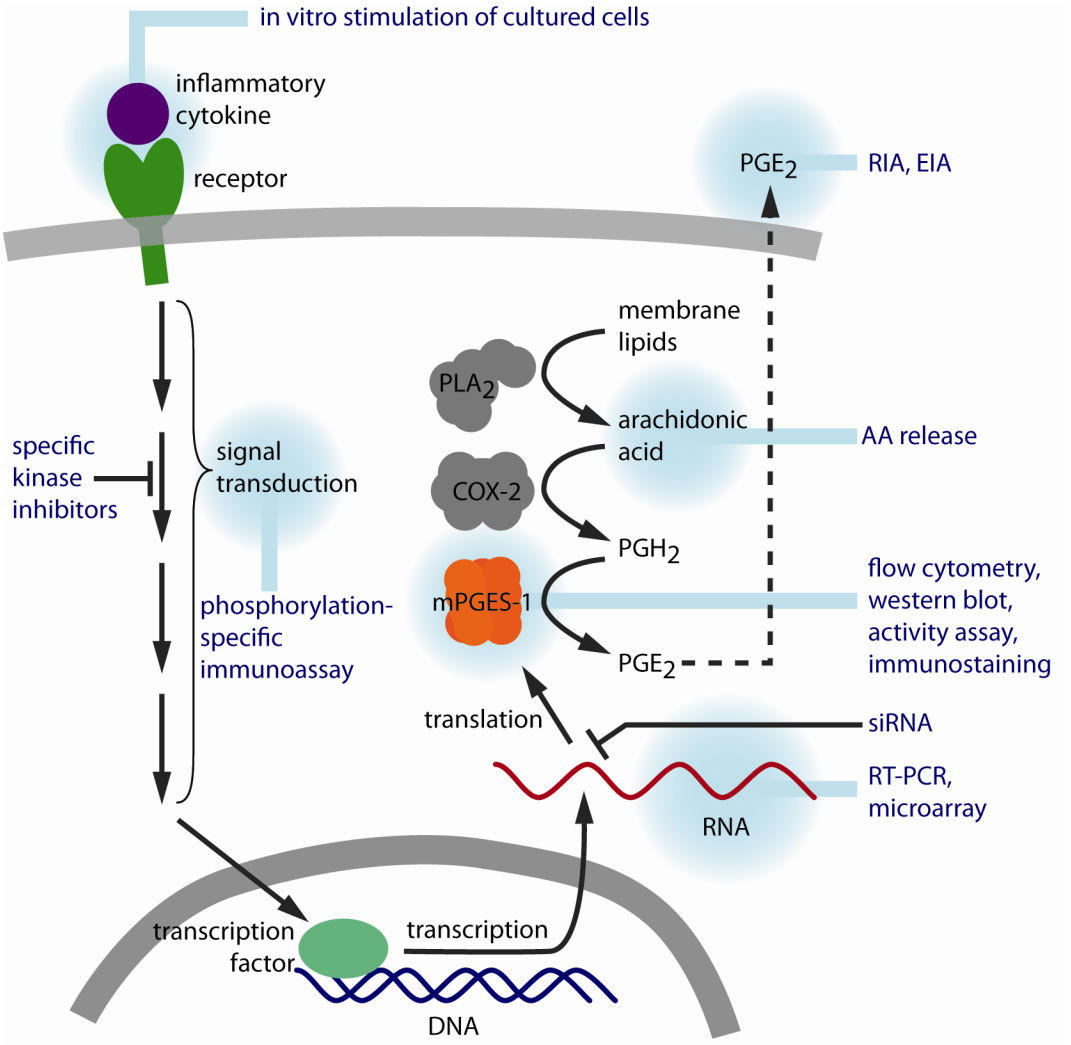

Figure 7. Schematic illustration of methods used in this thesis. The methods are described in detail in the published papers (I-IV). In this illustration, mPGES-1 is used as an example, but the other PGE synthases and COX-2 were also analysed.
Abbreviations: EIA, enzyme immunoassay; RIA, radioimmunoassay; siRNA, small interfering RNA; RT-PCR, reverse transcription polymerase chain reaction

Gingival tissues

One way to investigate the inflamed gingival tissue is to remove biopsies for study. The gingival biopsies (approximately 2×2×2 mm) are sectioned into thin slices, which are mounted on microscope slides. Once mounted on slides, the biopsy sections can be stained with fluorescently labelled or enzyme linked antibodies, which bind specifically to the protein of interest. Together with stainings that reveal the tissue structure, these analyses of tissue sections provide us with information relating to whether and where our proteins of interest can be found *in vivo*.

Gingival tissue collection (Study I)

A total of 11 gingival tissue biopsies were obtained from adult patients (age 35-68) diagnosed with periodontitis but otherwise healthy. The inclusion criteria were clinical signs of periodontitis at the site of biopsy collection, including radiographic bone resorption, pocket probing depth of ≥ 6 mm and bleeding on probing. Biopsies were taken during surgery as part of the normal course of periodontal therapy. The study was approved by the ethical committee at Karolinska Institutet. Each patient gave his/her informed consent for the use of gingival tissue.

Histological and immunohistochemical analysis (Study I)

Paraffin-embedded gingival tissues were sectioned (4 µm) and sections of each biopsy were stained with haematoxylin and eosin, as well as Giemsa, in order to assess the orientation of the epithelium and the degree of cell infiltration. To investigate leukocyte infiltration in the biopsies, tissues were stained using antibodies against the CD45 surface antigen. To further assess the degree of inflammation, tissues were also stained with antibodies against the inflammatory cytokine IL-1β. The details of these staining procedures can be found in the methods section in Study I.

For the immunostaining of PGE synthases and COX-2 in the biopsies, the sections were deparaffinised using xylene and were then rehydrated. Immunohistochemical staining was performed using primary antibodies for mPGES-1, mPGES-2, cPGES or COX-2. For negative controls, the primary antibody was substituted with an isotype-matched control antibody. After incubation with primary antibody, a biotinylated secondary antibody was added to the slides. The primary antibodies were mouse or rabbit IgG, while the secondary antibodies were anti-mouse or anti-rabbit, respectively. The biotinylation of the secondary antibody enabled streptavidin-conjugated horseradish peroxidase (HRP) to bind to the antibody complex. Addition of the HRP substrate 3,3' diaminobenzidine then resulted in the formation of a brown-coloured product in areas where the protein of interest was expressed. The slides were then mounted and observed under a light microscope.

To identify mast cells in the tissue, sections were deparaffinised and stained either with 0.5% toluidine blue in McIlvaine's buffer (pH 4) or with anti-human mast cell tryptase, using an automated instrument.

Immunofluorescent double staining (Study I)

Gingival tissues were immediately snap-frozen in liquid nitrogen, and then cryostat sectioned (7 µm). The sections were then fixed, permeabilised and blocked. After blocking, the slides were incubated with a mixture of two primary antibodies consisting of anti-mPGES-1 (polyclonal rabbit) and antibodies targeting the fibroblast marker anti-prolyl-4-hydroxylase, the endothelial cell marker von Willebrand factor, or smooth muscle actin (all monoclonal mouse). After washing, the sections were incubated with a mixture of secondary antibodies conjugated with fluorophores. The anti-rabbit secondary antibody was conjugated with Alexa Fluor 594 (red) and the anti-mouse secondary antibody was conjugated with Alexa Fluor 488 (green). The slides were washed, mounted and observed using a fluorescence microscope and image analysis was performed with the NIS-Elements software package. For all experiments, isotype-matched irrelevant antibodies were used as negative controls in the staining procedures.

Cell cultures

Cell cultures are useful model systems that serve as our substitute for the real live tissue of a real live patient. Cell cultures naturally lack characteristics that define the tissue, such as several interacting cell types, a tissue structure and an extracellular matrix. On the other hand, the cell culture model system readily lends itself to experimentation which may be neither ethical nor practical to perform on patients. Using cell cultures, the reactions of a specific cell type to a specific set of stimuli can be studied in isolation, thereby facilitating the elucidation of the complex network of interactions that contribute to the process of chronic inflammation. This section describes the cell culture of primary human gingival fibroblasts, as well as our treatment of the cells with a wide range of biological and chemical substances affecting the cells in various ways.

Gingival fibroblasts (Studies I-IV)

Human gingival fibroblasts were established from gingival biopsies obtained from healthy patients with no clinical signs of periodontal disease. Minced pieces of gingival tissue were explanted to tissue culture flasks containing culture medium including serum and antibiotics. Gingival fibroblasts were obtained by trypsinisation of the primary outgrowth of cells from the minced pieces of gingival tissue. The cells were then grown at 37°C with 5 % CO_2 and routinely passaged by trypsinisation.

Gingival fibroblasts were seeded in Petri dishes or cell culture plates, using culture medium supplemented with serum, and cultured for 24 h at 37°C. The cell layers were then rinsed with serum-free culture medium followed by the treatment of the cells with one of the inflammatory cytokines IL-1β or TNFα, in the presence or absence of substances summarised in Table 1. The cells were incubated for different time periods, ranging from 10 minutes to 24 h. In Study I, only IL-1β or TNFα were used, on different cell types. In Study II, the cells were treated with the inflammatory cytokines in combination with MK-886 or as described under "Knock-down of mPGES-1 by small interfering RNA (siRNA)" below. In Study III, various chemicals affecting intracellular signalling pathways were used, as well as components of the prostaglandin pathway. In Study IV, TNFα was used in a time series of experiments for microarray analysis, followed by further time-series experiments, including inhibitors of the JNK and NF-κB signalling pathways. After

		Used in study			
Name	**Effect**	**I**	**II**	**III**	**IV**
IL-1β	inflammatory cytokine	X	X	X	X
TNFα	inflammatory cytokine	X	X	X	X
MK-886	mPGES-1 inhibitor		X		
dexamethasone (Dex)	anti-inflammatory glucocorticoid		X	X	
15-deoxy-Δ (12,14)-prostaglandin J_2 (15d-PGJ_2)	anti-inflammatory prostaglandin			X	
4-bromophenacyl bromide (BPB)	PLA_2 inhibitor			X	
arachidonic acid (AA)	prostaglandin precursor			X	
bisindolylmaleimide (BIS)	PKC inhibitor			X	
PD 153035 hydrochloride (PD)	tyrosine kinase inhibitor			X	
phorbol-12-myristate-13-acetate (PMA)	PKC activator			X	
prostaglandin E_2 (PGE_2)	inflammatory mediator			X	
prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)	prostaglandin downstream of PGE_2			X	
pyrrolidine dithiocarbamate (PDTC)	NF-κB inhibitor			X	
SB 203580 (PD)	p38 MAPK inhibitor			X	
Bay 11-7082 (Bay)	NF-κB inhibitor				X
Ro 106-9920 (Ro)	NF-κB inhibitor				X
SP600125 (SP)	JNK inhibitor				X

Table 1. Substances used to treat gingival fibroblasts. The substances in this table were used to treat the cells in Studies I-IV.

treating the cells for the different incubation periods, culture medium was removed and stored at -20°C for subsequent analysis. The cell monolayer was washed and cells were collected for flow cytometric analysis, lysed for protein isolation, or lysed for isolation of total RNA.

Smooth muscle cells, endothelial cells, mast cells and mouse gingival fibroblasts (Study I)

The main cell type used for the *in vitro* experiments throughout this thesis is human gingival fibroblasts (Studies I-IV). For the isolation and culture procedures of human umbilical vein endothelial cells, human airway smooth muscle cells, mast cells and mPGES-1 knockout mouse gingival fibroblasts, the reader is referred to the methods section in Study I.

Co-cultures (Study I)

In Study I, cells were also co-cultured with lymphocytes isolated from whole blood, as an *in vitro* model of inflammation. Fibroblasts, endothelial cells or smooth muscle cells were seeded in Petri dishes. The cells were rinsed and cultured either alone or in co-culture with lymphocytes suspended in culture medium. The lymphocytes grow in suspension, whereas the other cells adhere to the surface of the Petri dish. Both direct cell-to-cell contact and interaction by soluble mediators is possible between the cell types in this co-culture system. After 18 hours of incubation, the co-culture medium containing lymphocytes was removed and centrifuged, after which the medium was collected and stored at -20°C for PGE_2 measurements. Fibroblasts, endothelial cells or smooth muscle cells were trypsinised and all cell types were collected and analysed by flow cytometry.

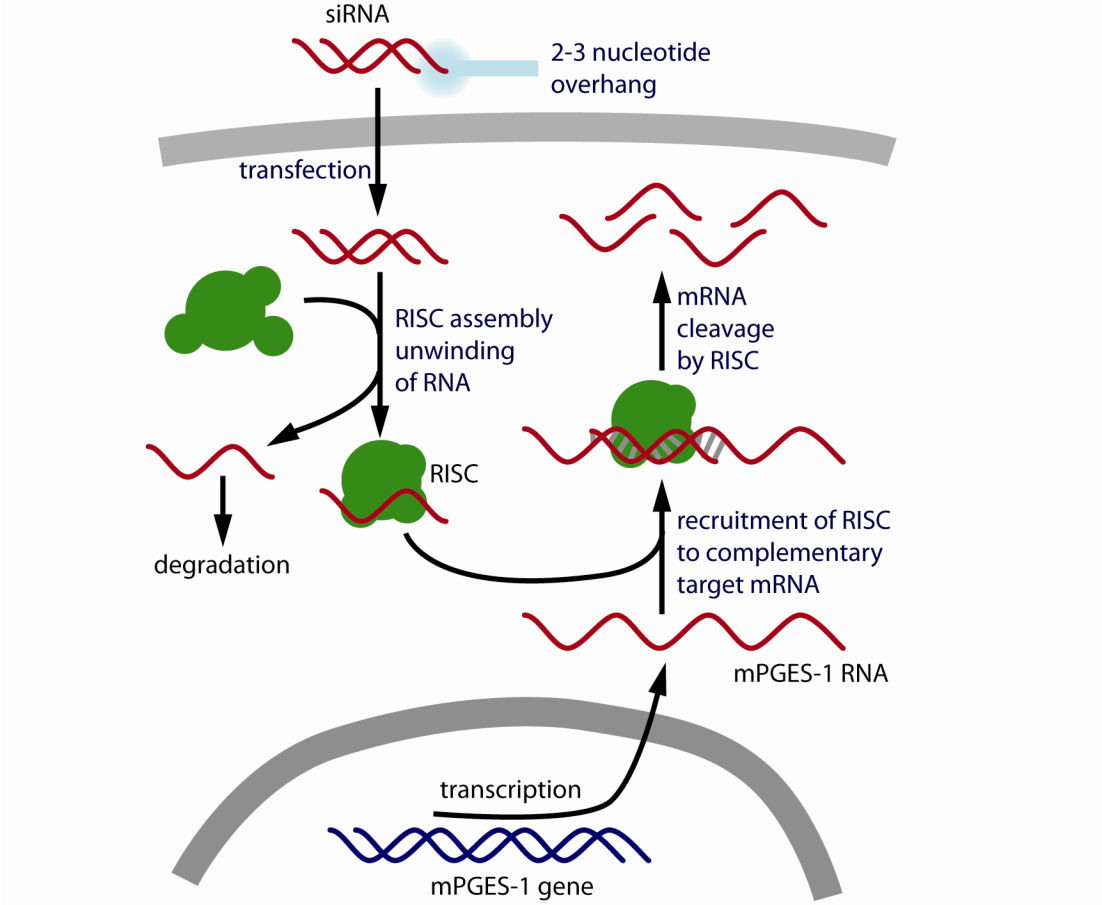

Figure 8. The mechanism of gene knock-down by small interfering RNA (siRNA). The RNA-induced silencer complex (RISC) is assembled. One strand of the double-stranded RNA is integrated into RISC and the other strand is degraded. The RISC with a bound siRNA binds to the complementary mRNA and cleaves it, thereby preventing translation, i.e. the protein expression of the target gene. A 2 to 3 nucleotide overhang is needed for the siRNA to be recognised by the RISC.

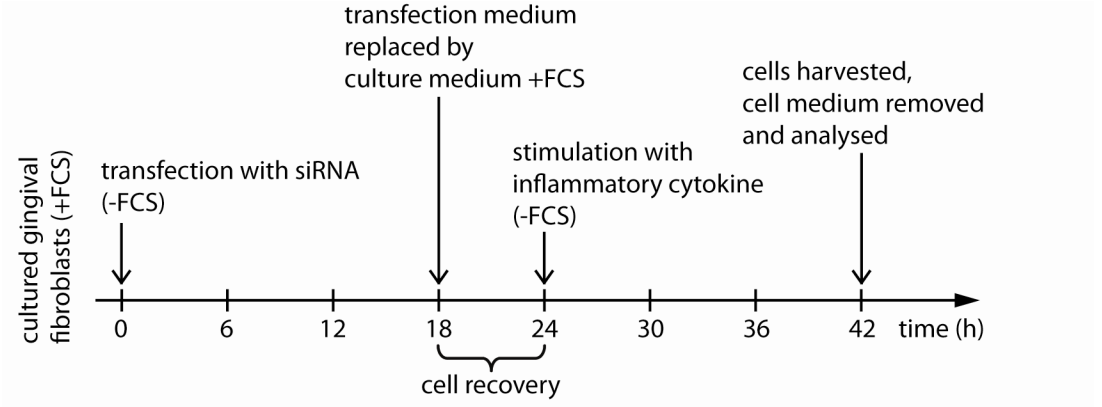

Figure 9. Laboratory procedure for siRNA experiments. Transfection with siRNA targeting mPGES-1 was performed to knock down mPGES-1 expression. Control cells were mock transfected without siRNA.

Abbreviations: FCS, fetal calf serum; mPGES-1, microsomal prostaglandin E synthase-1; siRNA, small interfering RNA.

Knock-down of mPGES-1 by small interfering RNA (siRNA) (Study II)

The method of siRNA gene knock-down is based on a mechanism which is present in many eukaryotic species to regulate endogenous genes and to defend the genome from invasive nucleic acids.[212] The mechanism of gene knock-down, as illustrated in Figure 8, is dependent on the assembly of the RNA-induced silencer complex (RISC). One strand of the double-stranded RNA is integrated into RISC and the other strand is degraded. The RISC with a bound siRNA recognises the complementary mRNA and cleaves it, thereby preventing translation and effectively knocking down the protein expression of the target gene. In Study II in this thesis, siRNA was introduced into the cells using a transfection reagent. The transfection reagent incorporates the siRNA into vesicles which can then fuse with the cell membrane to enable the siRNA to enter the cytoplasm. The laboratory procedure for siRNA experiments is outlined in Figure 9. Briefly, gingival fibroblasts were transfected with siRNA targeting mPGES-1 or mock transfected without siRNA. To allow the cells to recuperate after the transfection, a recovery period of 6 h was then allowed before the cells were stimulated with either of the cytokines TNFα or IL-1β. After stimulation, culture medium was removed and stored at -20°C for subsequent PGE_2 determination and the cells were harvested for flow cytometric, western blot or activity analysis.

RNA expression analyses

In each human being, the DNA, our genome, is the same in a fibroblast of the gingiva and, for instance, a neuron of the brain. The difference between the cells is accounted for to a large extent by differences in gene expression; i.e. the actual "building" of the proteins from the "blueprints" of the genome. Genes that are not transcribed into mRNA and subsequently translated into protein are unable to perform a function in the cell. There are several different methods for measuring gene expression. For the analysis of mRNA levels, the crucial improvement of the polymerase chain reaction (PCR) by Nobel laureate Kary B. Mullis and co-workers in the mid-1980s[213,214] allowed for its use in molecular biology and opened up a whole new world of opportunities. With the ability to amplify the amount of a specific DNA sequence to a level at which it is readily detectable, mRNA, converted to complementary DNA (cDNA), could be visualised using agarose gels and ethidium bromide. Another breakthrough in the analysis of mRNA gene expression was the first microarray, which was described in 1995.[215] This first microarray consisted of 45 gene transcripts spotted on a glass slide, but today the number of spots on a microarray chip can be three orders of magnitude larger. Microarray platforms enable the simultaneous analysis of mRNA gene expression using a comparatively small amount of sample. The two methods, reverse transcription PCR and oligonucleotide microarray analysis, are used for the analysis of mRNA expression in this thesis. However, the development of new techniques is ongoing and our group and others now rely progressively on sequencing methods for gene expression profiling.

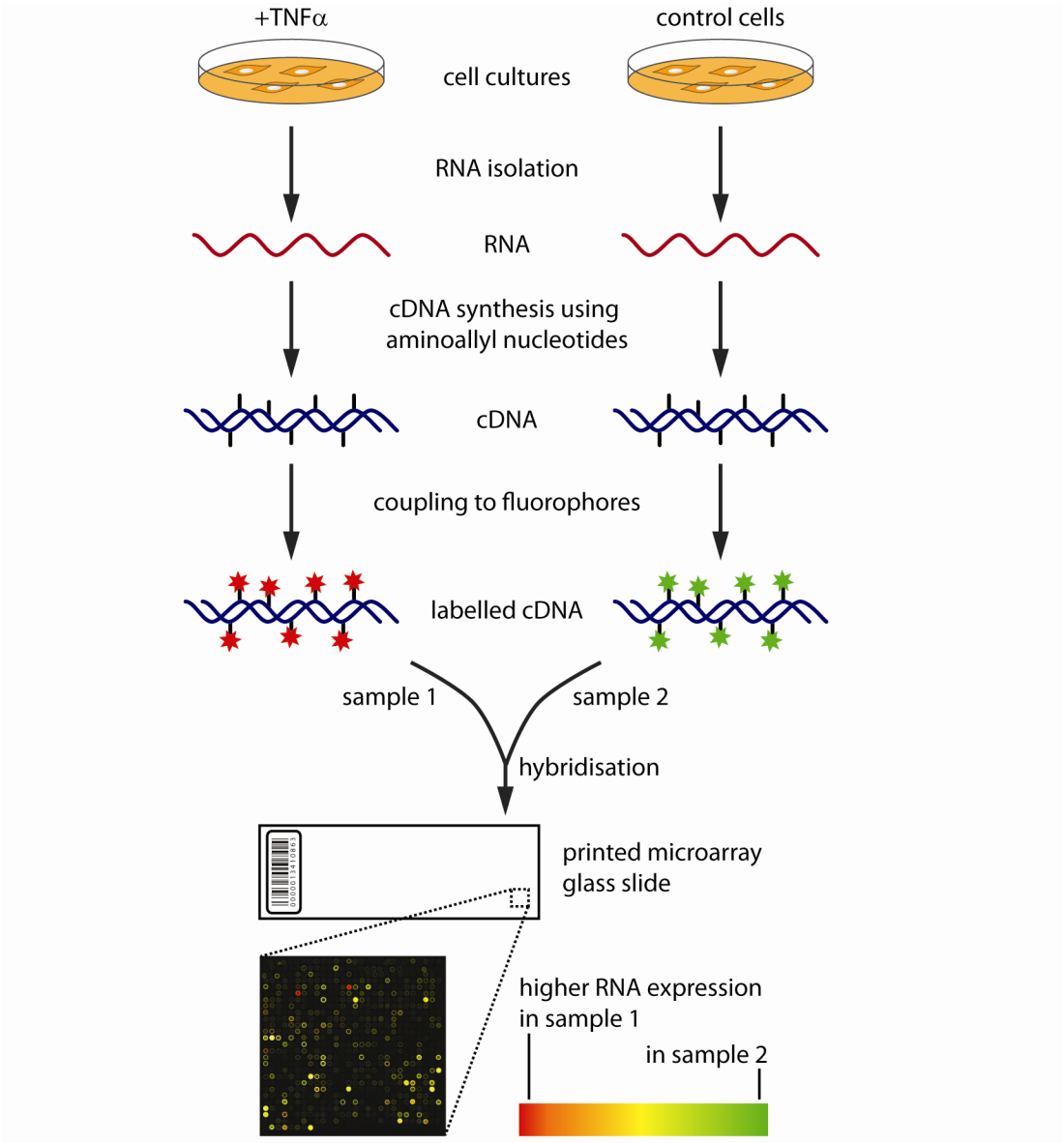

Figure 10. Schematic illustration of the process of microarray analysis. This illustration shows one hybridisation between TNFα-treated cells and control cells. In the study design used in this work, each sample was hybridised four times in different combinations, as shown in Figure 11.

RNA isolation and reverse transcription PCR (Studies II and III)

Gingival fibroblasts were seeded in Petri dishes and grown as described under the "cell culture" section. After 6 h of incubation with or without treatment, total RNA was isolated from gingival fibroblasts using the commercially available RNeasy kit and quantified spectrophotometrically at 260/280 nm. To obtain first-strand cDNA, RNA was reverse transcribed using the enzyme reverse transcriptase. The cDNA was then used as a template for PCR amplification. As a control, the housekeeping gene GAPDH was also amplified. From each PCR reaction product, 10 µl was separated on an agarose gel with ethidium bromide, together with a DNA-ladder standard. Ethidium bromide binds to DNA and fluoresces when irradiated with UV light, thereby enabling the visualisation of the PCR products. For each experiment, PCR amplifications without cDNA were performed as negative controls.

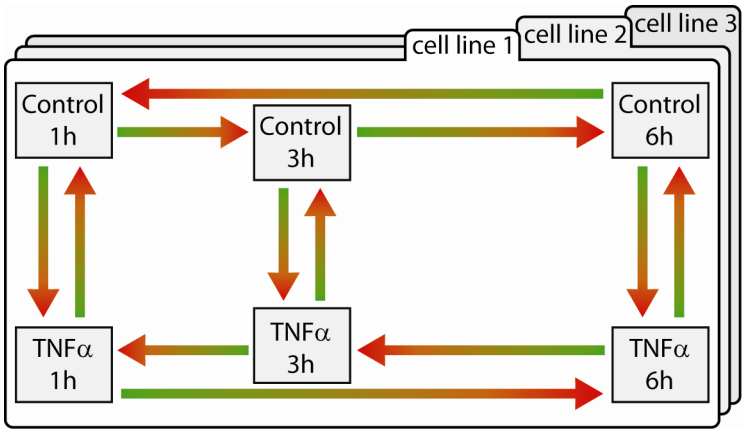

Figure 11. Study design for microarray experiments. Each arrow corresponds to one hybridisation where the green sample is labelled with Cy3 and the red sample with Cy5. Samples are from cells treated with TNFα or from control cells, incubated for the indicated times. The same design was repeated for each of three separate cell lines of primary human gingival fibroblasts.

Microarray analysis (Study IV)

In a microarray analysis, the mRNA expression of a large number of genes is analysed simultaneously. The microarray itself looks like a regular glass slide but contains tens of thousands of tiny spots of DNA. In the microarray chip used in this analysis, each spot, or feature, contains a large number of copies of a single 70 bp long sequence of DNA, which maps to a specific gene of the human genome. There are 35,344 features representing 28,948 Entrez Gene IDs[216] of which 17,972 are unique. The microarrays were printed at the KTH microarray core facility.

To obtain samples for microarray analysis, three separate cell lines of gingival fibroblasts were seeded in Petri dishes. After incubation for 1, 3 or 6 h with or without TNFα, the cells were immediately frozen in liquid nitrogen and then stored at -70°C for the subsequent isolation of total RNA. Total RNA was isolated from fibroblasts using the commercially available RNeasy kit and quantified spectrophotometrically at 260/280 nm. Synthesis of cDNA was then performed using the RNA from each sample as template. In the cDNA synthesis reaction, aminoallyl nucleotides were used and, after the cDNA synthesis, these were coupled to fluorophores, thus fluorescently labelling the samples. Two fluorophores were used, Cy3 (red) and Cy5 (green). To analyse the cDNA using the microarray, the samples are hybridised in pairs (one green and one red) to each microarray slide, as illustrated in Figure 10. The cDNA of the samples then bind to each spot on the array and the sample with the highest expression of a particular gene subsequently has the largest amount of fluorescently labelled cDNA bound to that spot.

After the hybridisation step, the microarray slides are scanned, resulting in two images – one red image, where the different intensities of Cy3 are shown for each spot, and one green image for Cy5. The images are combined and analysed using image analysis software which produces a ratio between red and green intensities for each spot. Statistical methods are then used to combine the data from all hybridisations and identify differentially expressed genes in different comparisons. A microarray experiment can be set up in many different ways, using different hybridisation combinations of the samples. For Study IV we chose a time-course factorial study design (Figure 11), optimised to estimate the interaction effect between TNFα treatment and time.[217]

The statistical analysis of microarray data was performed using the open source software R,[218] using different software packages, including the KTH package and packages from the Bioconductor open source software project for analysis of genomic data.[219] Four filters were

first used to remove spots with abnormal physical properties. On average, 75% of all spots passed the filters for each slide. After filtering, the slides were normalised using print tip Lowess normalisation and a moderated t-test was used to identify differentially expressed (DE) genes.[220,221] A false discovery rate algorithm was then applied to correct for multiple testing.[222] Thereafter, differentially expressed genes were defined as genes with a q-value (the false discovery rate analogue of a p-value) of < 0.05, meaning that the proportion of false positives among the differentially expressed genes was 0.05.

To discern the patterns present within the DE genes in our dataset, we performed an enrichment analysis, using the Gene Ontology (GO) database.[223] The GO database is a structured controlled vocabulary (ontology) describing gene products in terms of their associated biological processes, cellular components and molecular functions. We used GO to assign functions to the DE genes in our dataset.[223] We then utilised the GO annotation in performing an enrichment analysis to discover biological themes among the DE genes in the different comparisons.[224] In the enrichment analysis, groups of genes linked to a particular GO term, such as "immune response", are analysed to see whether more of the genes in that group are DE in our dataset than would be expected by chance. This analysis enables us to obtain an overview of the vast amounts of data obtained from the series of microarray analyses.

Protein expression analyses

The analysis of mRNA levels is a very useful method for detecting gene expression. To analyse a specific gene, good primers for the PCR reaction is the main requirement. Furthermore, the analysis of mRNA levels is readily multiplexable. However, what generally matters most in the cell is the amount of protein translated from the mRNA in question. For enzymes like the PGE synthases, the protein is what performs the actual catalysis of the reaction. However, protein levels are commonly more difficult to determine than mRNA levels. All the approaches used to measure protein levels in this thesis are based on antibodies. Antibodies are large Y-shaped proteins used by our immune systems to identify and neutralise pathogens such as bacteria or viruses. Antibodies that bind specifically to a particular protein can be produced and used in research applications. Polyclonal and monoclonal antibodies are used in the studies included in this thesis for western blotting and flow cytometric analyses, as well as for tissue staining (as described in the "Histological and immunohistochemical analysis" section). Primary antibodies are used to bind specifically to the protein of interest. Then, to detect the primary antibodies, secondary antibodies are allowed to bind to the primary antibodies. These secondary antibodies are conjugated to a reporter, often a fluorophore or an enzyme, such as HRP, which is able to catalyse a reaction forming a coloured product. HRP can also be used to catalyse a reaction forming a light-emitting product (chemiluminescence), the presence of which can be recorded on a photographic film.

Western blotting (Studies I-IV)

Cells were seeded and grown as described under the "cell culture" section. To isolate the total protein, the cells were washed with PBS and resuspended in lysis buffer containing protease inhibitors. The protein concentration of the cell lysates was determined and equal amounts of the obtained protein were separated by electrophoresis on a 4-15% linear gradient polyacrylamide tris-HCl gel or a 10% sodium dodecyl sulphate-polyacrylamide

gel. The protein was then transferred to a nitrocellulose membrane which was blocked and then incubated over night with primary antibody (mPGES-1, mPGES-2, cPGES or COX-2). Following primary antibody incubation, the membranes were washed and then incubated with HRP-conjugated secondary antibody. The membranes were washed, developed using chemiluminescence and exposed to hyperfilm-ECL.

Flow cytometry (Studies I, II and IV)

Cells were seeded and grown as described in the "cell culture" section. After treatment, the cells were collected by trypsinisation and/or centrifugation of culture medium depending on cell type and washed with PBS. Thereafter, the cells were fixed in paraformaldehyde and permeabilised using saponin. Saponin allows the antibodies to pass through the cell membrane, enabling the staining of intracellular proteins like the PGE synthases. The cells were then incubated with primary antibodies for mPGES-1, mPGES-2, cPGES or COX-2. After incubation and washing, the cells were further incubated with a secondary, fluorescently labelled antibody. Cells could then be analysed in a flow cytometer for the expression of mPGES-1, mPGES-2, cPGES or COX-2. The flow cytometer analyses each cell separately, recording the forward scatter (giving information on cell size) and the side scatter (pertaining to cell granularity), as well as the fluorescence at different wavelengths. The forward and side scatter parameters in this setting serve to confirm that we have only one cell type in our sample. The fluorescence intensity is related to the expression of the analysed protein. The results obtained are shown as histograms of cell counts, drawn using the program R, together with the software package rflowcyt,[218,219] or presented as mean fluorescence intensity.

Activity analyses

Although the amount of a specific enzyme may have a great impact on its effect on the cell, the activity of an enzyme can be even more important. Taking protein kinases as an example, these enzymes lie dormant in the cell until they are activated, for instance by phosphorylation. The activated kinase is then able to phosphorylate other downstream enzymes, in turn activating them. This process results in intracellular signalling cascades which are much faster than would be possible if the signal depended on the *de novo* synthesis of the enzymes involved. However, the amounts of enzyme present before activation can still affect the strength of the signal.

Phosphorylation-specific analysis (Study IV)

To confirm that certain intracellular signalling pathways were activated by TNFα treatment of the cells, and also to confirm the inhibition of these signalling pathways by specific inhibitors, we used antibodies that specifically bind only to the phosphorylated, i.e. activated, form of the enzyme. Since kinase phosphorylation is such a rapid process, both short and long incubation times were used. Cells were seeded and grown in Petri dishes as described above. After an incubation period of 10 minutes, 1 h, 3 h, 6 h or 24 h, the cells were scraped in PBS and centrifuged. The pellet was then resuspended in lysis solution, containing protease and phosphatase inhibitors, and frozen at 20°C. The samples were then thawed and centrifuged and the supernatant was collected. The lysate protein concentration was determined, followed by the addition of an equal volume of assay buffer from the Bio-Plex phosphoprotein detection kit. The samples were then frozen at -20°C

until determination of the amount of phosphorylated JNK or NF-κB using Luminex technology on a Bio-Plex Suspension Array System.

PGE synthase activity assay (Study II)

The function of the PGE synthase enzymes is to convert PGH_2 to PGE_2. This reaction can be performed outside the cell as an *in vitro* enzyme assay, to evaluate the PGE synthase activity in the cell lysate from a specific cell culture experiment. To achieve this, gingival fibroblasts were seeded in Petri dishes and treated with TNFα or IL-1β, or as described under "knock-down of mPGES-1 by small interfering RNA (siRNA)". After treatment, the cells were collected by trypsinisation and were then lysed by freeze-thawing and sonication. In the activity assay, equal amounts of protein from each sample were incubated with PGH_2 in Tris buffer supplemented with glutathione. The reaction was allowed to proceed for 60 s at room temperature and was thereafter terminated by the addition of $FeCl_2$ which decomposes the remaining PHG_2 to non-relevant metabolites.[225] Following centrifugation, the supernatants were collected and frozen (-20°C) for subsequent PGE_2 analysis. For negative controls, the reaction was run without cell lysate.

Analyses of culture media

The cells in our *in vitro* studies grow in culture medium. When we perform experiments, substances are added to the culture medium and these substances affect the cells. However, the cells also affect the culture medium by releasing a range of compounds in response to treatment. The medium that we collect after treatment of the cells can therefore give us information on the processes inside the cells, such as the production of PGE_2.

Prostaglandin analysis (Studies I-IV)

The radioimmunoassay (RIA) and enzyme immunoassay (EIA) are both antibody-based methods. The difference lies in the detection of the antibodies after they have bound to the molecule of interest, for example PGE_2. RIA uses radioactive labelling, which can be measured using a gamma counter. EIA uses an enzymatic reaction, resulting in a coloured product which can be measured spectrophotometrically. The amount of PGE_2 in the culture media was determined using an RIA kit, a conventional EIA kit or using Luminex technology on a Bio-Plex Suspension Array System with a commercially available EIA kit. The amount of $PGF_{2\alpha}$ in the culture media was determined using an EIA kit (Study II). The amount of PGE_2 produced in the activity assay (Study II) was measured using Luminex technology on a Bio-Plex Suspension Array System with a commercially available EIA kit.

Analysis of ^{3}H-arachidonic acid (^{3}H-AA) release (Study II)

Analysing AA release gives us information on the total amount of eicosanoids (prostaglandins and leukotriens) produced by the cell. It was used in conjunction with siRNA knock-down experiments in Study II. After the transfection and recovery of the cells, but before stimulation with cytokines, a 20-hour incubation period was introduced into the normal siRNA protocol (see Figure 9, page 24 for the siRNA protocol). During this incubation period, AA labelled with ^{3}H was added to the culture medium and thus incorporated into the cells. The cells were then stimulated with TNFα for 24 h after which the culture medium was collected and analysed for levels of ^{3}H using a scintillation counter. To obtain the total activity, cell layers were lysed and analysed using a scintillation

counter. The results were presented as ^{3}H release divided by total ^{3}H activity (medium and cells), relative to control cells. An increased value represents an increase in AA-derived products such as prostaglandins secreted by the cells. The activity of ^{3}H represents free ^{3}H-AA, as well as ^{3}H-labelled metabolites.

Statistical analyses

For statistical analyses other than those used for microarray data (Study IV), we used Student's t test (two-tailed). The t test was first introduced in the early 19th century by William Sealy Gosset who used "Student" as his pen name in order to hide the fact that he had developed the statistics as an aid to monitoring the quality of stout at the Guinness brewery in Dublin, Ireland. In this work, all cell culture experiments were analysed in triplicate and reproducible data representing one of at least three independent experiments were demonstrated. The results are expressed as the mean value ± standard deviation. p-values of less than 0.05 were considered statistically significant.

Results and discussion

The four studies included in this thesis investigate the expression and regulation of PGE synthases in periodontitis-affected gingival tissue and in gingival fibroblasts. Study I deals with the *in vivo* expression of PGE synthases in gingival tissue collected from patients with periodontitis, as well as the *in vitro* expression and regulation of the enzymes in various cell types. In Study II, a possible inter-regulation of the three PGE synthase isoforms is investigated by means of siRNA knock-down of mPGES-1 *in vitro* using human gingival fibroblasts, the predominant cell type in gingival connective tissue. Studies III and IV further explore the regulation of the inducible PGE synthase isoform, mPGES-1, in gingival fibroblasts. All four studies have been published in peer-reviewed journals and can be found in their entirety in the appendix. This section gives a brief overview of the results of these studies and a discussion of the findings in relation to the current literature.

Expression of prostaglandin E synthases in periodontitis-affected gingival tissue (Study I)

PGE_2 is a key mediator in several chronic inflammatory conditions including periodontitis.[91-94] A group of isoenzymes in the prostaglandin E cascade, the PGE synthases, catalyse the terminal step of PGE_2 biosynthesis. The *in vivo* expression of all three PGE synthase isoforms, mPGES-1, mPGES-2 and cPGES, has not been previously investigated in gingival tissue, although mPGES-1 has been shown to be expressed in gingivitis.[226] The upstream enzyme COX-2, which has been more thoroughly studied, has also been shown to be expressed in gingivitis as well as in periodontitis.[145,226] We studied the expression of PGE synthases in inflamed gingival tissue from patients with periodontitis, finding the protein expression of the isoforms mPGES-1, mPGES-2 and cPGES, as well as COX-2, in epithelium, fibroblasts, endothelial cells, vascular smooth muscle cells and inflammatory cells. No previous data on the expression of mPGES-2 or cPGES in inflamed gingiva have been published. However, our results relating to the *in vivo* localisation of mPGES-1 and COX-2 are consistent with previous studies showing the positive staining of these inducible enzymes in epithelial, endothelial, and fibroblast-like connective tissue cells of the gingiva.[226,227] These results from Study I are also in accordance with findings in synovial tissue from patients with rheumatoid arthritis[228] and gastric ulcer tissue[158], emphasising the importance of the PGE synthases in diverse chronic inflammatory conditions. Our finding that all three PGE synthases are expressed in inflamed gingival tissue from patients with periodontitis suggests that all three enzymes may contribute to PGE_2 synthesis in gingival tissue. We then proceeded to investigate the regulation of PGE synthases *in vitro*, further exploring their role in inflammation-induced PGE_2 synthesis.

Cellular regulation of PGE_2 production (Study I)

Levels of PGE_2 have been shown to be enhanced in gingival tissue from patients with periodontitis, highlighting PGE_2 as a key inflammatory mediator involved in this chronic inflammatory condition.[92] We showed that all three PGE synthases are widely expressed in gingival tissue from patients with periodontitis. To further explore the cellular regulation of the PGE synthases and PGE_2 production in gingival connective tissue, we used cell cultures stimulated with the inflammatory cytokines TNFα or IL-1β as an *in vitro* model of inflammation in terms of PGE_2 synthesis. The cell cultures used as *in vitro* models were primary human gingival fibroblasts, endothelial cells (human umbilical vein endothelial cells, HUVEC, of the CRL-1730 cell line), smooth muscle cells (primary human airway smooth muscle, HASM, cells) and mast cells (the HMC-1.2 human mast cell line and primary human cord blood mast cells). The results showed the protein expression of mPGES-1, mPGES-2, cPGES and COX-2, as well as basal levels of PGE_2 production, in cultured gingival fibroblasts, endothelial cells, smooth muscle cells and mast cells. Moreover, the expression of mPGES-1 and COX-2 was increased by TNFα and IL-1β in fibroblasts and smooth muscle cells, whereas, in endothelial cells, only COX-2 was induced, in response to TNFα. The up-regulated expression of the PGE_2-synthesising enzymes mPGES-1 and COX-2 was accompanied by enhanced PGE_2 production in fibroblasts and smooth muscle cells and, to a lesser extent, also in endothelial cells. Epithelial cells were not included in the *in vitro* studies, since our focus was on gingival connective tissue. Previous studies have shown increased PGE_2 production in gingival epithelial cells after stimulation with bacterial components.[229,230]

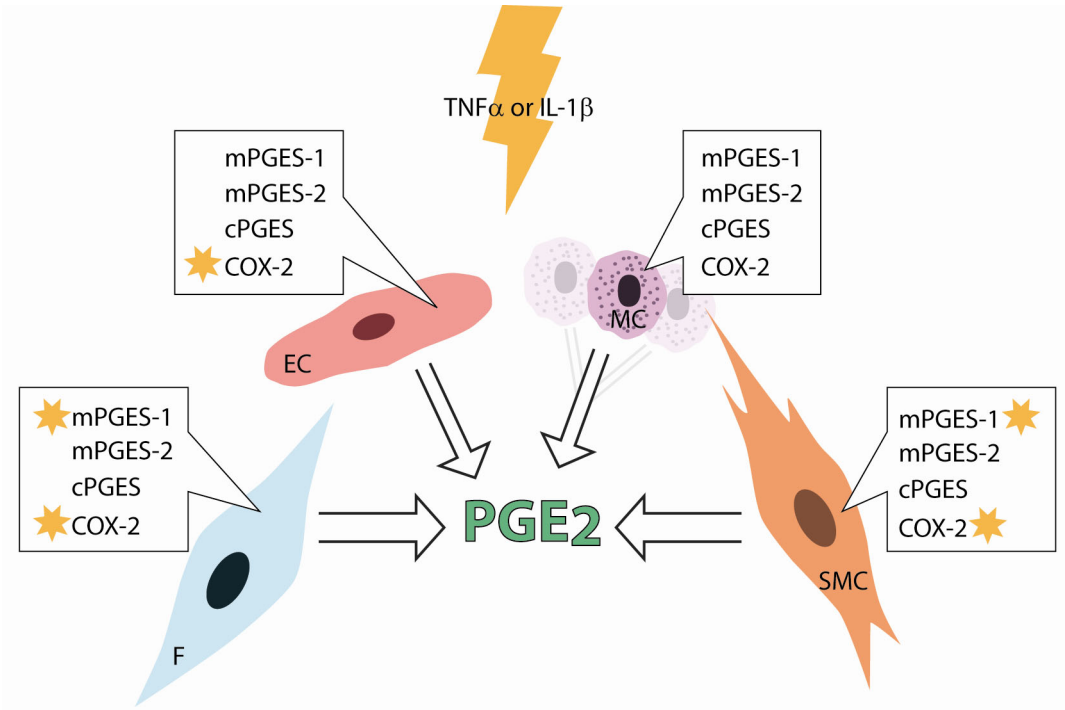

Figure 12. Schematic illustration of the results from Study I showing possible contribution of the cell types investigated to inflammation-induced PGE_2 production in gingival connective tisue. Stars signify increased expression in response to TNFα and/or IL-1β. Other cell types than those studied here most likely also contribute to the total PGE_2 production in gingival tissue.
Abbreviations: F, fibroblast; EC, endothelial cell; MC, mast cell; SMC, smooth muscle cell.

In cytokine-stimulated gingival fibroblasts, the observed increase in PGE_2 production via the enhanced expression of mPGES-1 and COX-2 is in agreement with data on synovial, orbital and gastric fibroblasts, as well as our previous data on gingival fibroblasts.[58,157,191,231-233] The information from our results, together with the above-mentioned studies of fibroblasts from other tissues, support the concept that fibroblasts play an important role in the inflammatory reaction instead of being solely involved in tissue homeostasis, as previously believed.[36,234] The facts that fibroblasts are prominent producers of PGE_2, as well as the most ubiquitous cell type in gingival connective tissue, further highlight these cells as possible attractive targets in the treatment strategy for periodontitis. The targeting of fibroblasts in the treatment of chronic inflammatory diseases has also been suggested by Flavell et al., who proposed that fibroblasts might be stimulated to produce pro-resolution mediators instead of inflammatory products.[36]

Regarding smooth muscle cells, our *in vitro* results showed that PGE_2 production and PGE_2-synthesising enzymes are up-regulated in HASM cells in response to TNFα and IL-1β. These results are in line with previous studies of primary human vascular smooth muscle cells, showing that mPGES-1 and COX-2 are induced by inflammatory cytokines.[175,235] Cultured endothelial cells, however, had only a modest basal production of PGE_2, which was slightly induced by TNFα but not by IL-1β. Up-regulation of COX-2 but not mPGES-1 expression accompanied the increase in PGE_2 production, which suggests that the mPGES-1 and COX-2 enzymes are regulated by different mechanisms in the endothelial cells. The issue of differential regulation of mPGES-1 and COX-2 expression will be further discussed in the section "Signal transduction pathways of cytokine-induced mPGES-1 expression in gingival fibroblasts". Our results regarding regulation of PGE_2-synthesising enzymes in endothelial cells are similar to previously

published data demonstrating the up-regulation of COX-2 in response to TNFα.[236] However, it has been reported that endothelial cells from saphenous vein do not express mPGES-1 mRNA, even after cytokine stimulation,[235] which may suggest a location-specific expression of PGE synthases in endothelial cells. The *in vitro* results showing the production of PGE_2 by the vascular cell types endothelial cells and smooth muscle cells suggest a possible role for these cells in inflammation-induced PGE_2 production *in vivo*. Although these vascular cells are not as ubiquitous in gingival tissue as fibroblasts, their location provides the first access to infiltrating immune cells and they may be among the earliest cells in the inflammatory cascade to start producing PGE_2.

The infiltration of inflammatory cells into the tissue is one of the hallmarks of gingival inflammation. The production of PGE_2 has been reported in inflammatory cells, such as monocytes,[237,238] lymphocytes[239] and PMNs,[240] but mast cells have been poorly studied in regard to the expression of PGE synthases and PGE_2 production. These cells were therefore included in the *in vitro* studies for the cellular regulation of PGE_2 and PGE_2-synthesising enzymes. Cultured mast cells produced low basal levels of PGE_2 and neither TNFα nor IL-1β treatment affected the PGE_2 production or the expression of PGE synthases or COX-2. However, mast cell numbers have been shown to be increased in inflamed gingival tissue,[241,242] suggesting that the basal PGE_2 production of these cells may still contribute to some degree to the increased PGE_2 levels in inflamed gingival tissue through an additive effect. To our knowledge, there are a very limited number of studies investigating the expression of PGE_2-synthesising enzymes or PGE_2 production in mast cells; however, increased COX-2 expression and PGE_2 production has been demonstrated in response to antigen stimulation and to the PLA_2 activator melittin, respectively.[243,244] Among the cells included in Study I, more likely contributors to inflammation-induced PGE_2 in periodontitis are the resident gingival fibroblasts and smooth muscle cells and possibly also endothelial cells. However, apart from the gingival fibroblasts, the cells used in our *in vitro* studies are not primary cells isolated from the human gingiva. The results should therefore only be taken as indications of possible *in vivo* mechanisms and our data warrant further confirmation also in cells isolated from periodontitis-affected gingival tissue.

In order more closely to mimic the *in vivo* situation, we used an *in vitro* model of inflammation in which cells were also co-cultured with lymphocytes. Co-culture of the cells with lymphocytes up-regulated mPGES-1 and COX-2 expression in parallel with increased PGE_2 production in fibroblasts and smooth muscle cells. One explanation for this up-regulation is the release of pro-inflammatory cytokines such as IL-1β from the lymphocytes,[245] stimulating the expression of PGE_2-producing enzymes and thereby resulting in increased PGE_2 production. The results relating to COX-2 up-regulation by lymphocytes in gingival fibroblasts are in line with previous results published by our group.[246] Collectively, these novel results represent a first step in the process of revealing the cellular source and mechanism behind increased PGE_2 in periodontitis. One possible scenario for the contribution of different cell types to inflammation-induced PGE_2 production in gingival connective tissue is depicted in Figure 12.

The role of mPGES-1 in cytokine-induced PGE_2 production (Studies I and II)

The results obtained from Study I indicated that gingival fibroblasts play an important role in inflammation-induced PGE_2 production. In the light of this, we are now going to delve more deeply into the regulation of PGE_2 production in these cells. The PGE synthase isoform mPGES-1 is generally considered to be the most inducible, inflammation-related enzyme of the three.[247,248] To further clarify the role of mPGES-1 in inflammation-induced PGE_2 synthesis, we used mPGES-1 null gingival fibroblasts derived from mPGES-1 knock-out mice.[249] These cells, isolated here for the first time, demonstrated markedly reduced PGE_2 production compared with cells from wild-type mice after treatment with TNFα (Study I). This indicates that the enzyme mPGES-1 is responsible for a large part of the cytokine-induced PGE_2 production. The small increase in PGE_2 production observed in TNFα-stimulated mPGES-1 null gingival fibroblasts may be due to the up-regulation of the upstream enzyme COX-2, together with the constitutively expressed mPGES-2 and cPGES. The impaired PGE_2 response demonstrated by our results is similar to data reported for mouse mPGES-1 null macrophages stimulated with LPS.[250]

Notably, it has been suggested that the second, glutathione-independent, membrane-associated PGE synthase, mPGES-2, may take over inflammation-induced PGE_2 synthesis in the event of a non-functional mPGES-1 enzyme.[183] To elucidate the hypothesis that mPGES-2 and/or cPGES may substitute for mPGES-1 in gingival fibroblasts, we investigated the possible up-regulation of mPGES-2 and cPGES, when mPGES-1 was knocked down by siRNA or inhibited using molecular inhibitors (Study II). Although the silencing of mPGES-1 using siRNA knocked down both TNFα- and IL-1β-stimulated mPGES-1 protein expression (39-77% and 54-79% knock-down respectively), no increase in mPGES-2 or cPGES expression was observed. The expression of the upstream COX-2 enzyme was likewise unaffected, suggesting that neither mPGES-2, cPGES nor COX-2 compensates for the down-regulated mPGES-1 expression. In spite of mPGES-1 knock-down, the production of PGE_2 was not affected. One explanation for this could be an alternative pathway for PGE_2 synthesis, involving the downstream prostaglandin $PGF_{2\alpha}$, since the levels of $PGF_{2\alpha}$ were increased by anti-mPGES-1 siRNA treatment. The prostaglandin $PGF_{2\alpha}$ can be synthesised from PGE_2 or directly from PGH_2, by distinct enzymes.[103] It is also possible for $PGF_{2\alpha}$ to be converted to PGE_2 by the enzyme PGE 9-ketoreductase[103] and thereby bypass the PGE synthases in PGE_2 production. The total release of AA, the intermediary for the synthesis of all prostanoids and leukotrienes (see Figure 4, page 8), was unaffected by mPGES-1 knock-down. It is possible that, when mPGES-1 is knocked down by siRNA, the COX-2 derived PGH_2 may be shunted towards $PGF_{2\alpha}$, which can in turn be converted to PGE_2 and thereby contribute to PGE_2 synthesis. This putative mechanism may partly explain the unaffected PGE_2 levels. Another possible reason for the unaffected PGE_2 production could be that the amount of cytokine-stimulated mPGES-1 that remains after knock-down (21-61% in our experiments) is enough to catalyse the conversion of all available COX-2-derived PGH_2 to PGE_2. In effect, this indicates that COX-2 is the rate-limiting enzyme in this setting. The hypothesis that COX-2 is the rate-limiting enzyme in the synthesis of PGE_2 has been put forward in several publications.[251-253] However, it has also been suggested that all three enzyme groups involved in PGE_2 biosynthesis can be rate-limiting,[169] and it is possible that, in case of a

more efficient knock-down of mPGES-1, this enzyme would become rate-limiting instead of COX-2.

To corroborate the siRNA results, the mPGES-1 inhibitor MK-886 was also used, resulting in effects similar to those obtained from mPGES-1 siRNA experiments: MK-886 reduced mPGES-1 expression without affecting mPGES-2, cPGES, COX-2 or PGE_2 production. In addition, the anti-inflammatory glucocorticoid dexamethasone, which is known to have a broad anti-inflammatory effect,[254] inhibited mPGES-1 and COX-2 expression, as well as PGE_2 production, without affecting mPGES-2 or cPGES expression.

Our results from the mPGES-1 knock-down experiments in Study II may suggest that PGE_2 production is more closely correlated to COX-2 expression than to mPGES-1 expression in gingival fibroblasts. However, the results from the mPGES-1 null fibroblasts in Study I show that the total abrogation of mPGES-1 expression does indeed dramatically reduce inflammation-induced PGE_2 production. Furthermore, the current knowledge of the side-effects of specific and non-specific COX-2 inhibitors[152,255] used to inhibit inflammation-induced PGE_2 production turns the spotlight on the PGE synthases. Taken together, this motivates further investigations into the endogenous regulation of mPGES-1 expression.

Signal transduction pathways of cytokine-induced mPGES-1 expression in gingival fibroblasts (Studies III and IV)

In Study I, we showed that fibroblasts may play a central role in producing inflammation-induced PGE_2 in gingival tissue. In Studies I and II, we further demonstrated that the most important of the three PGE synthases in this regard is mPGES-1, which, together with the upstream enzyme COX-2, is responsible for the increased production of PGE_2 when stimulated with TNFα or IL-1β. The next step was to investigate the intracellular signalling pathways that mediate the cytokine-induced up-regulation of mPGES-1 expression. For this reason, in Studies III and IV, the intracellular regulation of cytokine-induced mPGES-1 expression was studied in gingival fibroblasts. Inhibitors and stimulators of diverse signal transduction pathways were used in Study III to explore the complex network of interacting pathways involved in increased mPGES-1 expression.

To obtain a broader view of regulated signalling pathways in TNFα-stimulated gingival fibroblasts, a global gene expression profile was established in Study IV. Enrichment analysis of the vast amount of gene expression data led to the further investigation of signalling pathways NF-κB and JNK, using specific inhibitors and phosphorylation-specific immunoassays. The results of Studies III and IV indicated that the JNK and NF-κB pathways are involved in the signal transduction of cytokine-induced mPGES-1 expression in gingival fibroblasts. In Study III, the signalling pathways PKC, tyrosine kinase and p38 MAP kinase were ruled out as being involved in the signal transduction leading to increased mPGES-1 production. However, all three of these pathways were shown to be involved in COX-2 regulation, together with the NF-κB and JNK pathways. These results of Studies III and IV are summarised and illustrated in Figure 13. Although the JNK and NF-κB pathways are involved in both mPGES-1 and COX-2 up-regulation, the differences relating to PKC, tyrosine kinase and p38 MAP kinase indicate a lack of strict co-regulation of mPGES-1 and COX-2 in gingival fibroblasts. Bearing in mind the differential regulation of these enzymes by TNFα in endothelial cells (Study I), mPGES-1 and COX-2 regulation might be uncoupled in several cell types, including the endothelial

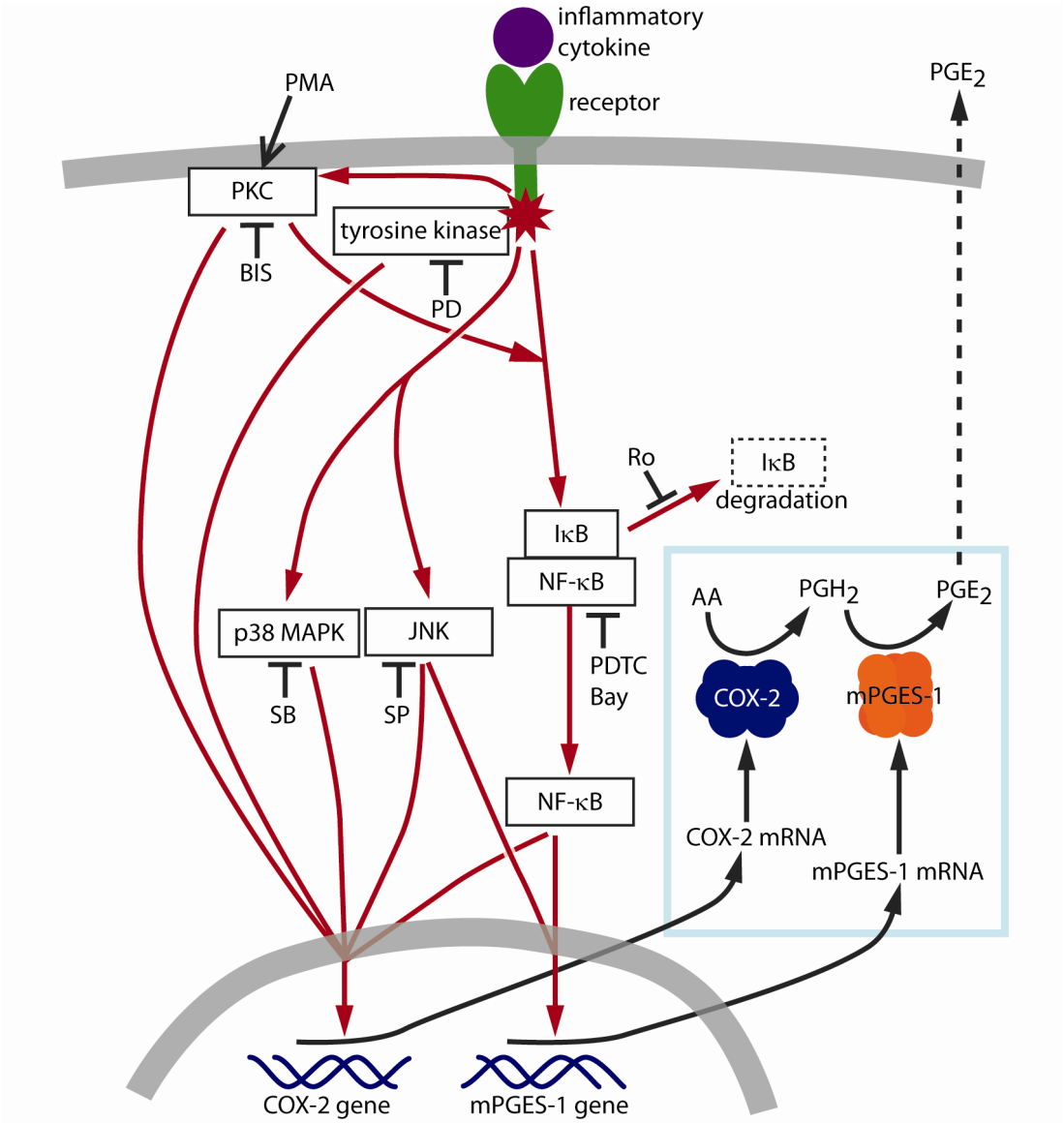

Figure 13. Schematic illustration of the results from Studies III and IV regarding signal transduction pathways involved in the regulation of mPGES-1 and COX-2 expression in human gingival fibroblasts. The kinases and transcription factors depicted are those that are included in the present work, and intermediate signalling steps are not shown. There may be additional cross-talk between signal pathways, and other pathways than those depicted here may also be involved in the regulation of cytokine-induced mPGES-1 and COX-2.

Abbreviations: AA, arachidonic acid; Bay, Bay 11-7082; BIS, bisindolylmaleimide; COX, cyclooxygenase; IκB, inhibitor of NF-κB; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; mPGES-1, microsomal prostaglandin E synthase-1; p38 MAPK, p38 mitogen-activated protein kinase; PD, PD 153035 hydrochloride; PDTC, pyrrolidine dithiocarbamate; PGH_2, prostaglandin H_2; PGE_2, prostaglandin E_2; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; Ro, Ro 106-9920; SB, SB 203580; SP, SP600125.

cells and gingival fibroblasts investigated in this work. A lack of co-regulation between mPGES-1 and COX-2 has also been reported in LPS-stimulated rat microglia, further supporting this suggestion.[194]

Our novel finding that JNK is involved in TNFα-induced mPGES-1 expression is in line with results for IL-1β-stimulated cells, such as cardiac fibroblasts and A549 human lung epithelial cells.[160,195] Additionally, JNK has been implicated in the chronic inflammatory conditions rheumatoid arthritis and inflammatory bowel disease.[256,257] The results presented here indicate that this pathway may also be important in periodontitis,

perhaps even as a possible therapeutic target for chronic inflammation, as suggested for rheumatoid arthritis based on the results of rodent models.[258]

Our finding that the NF-κB pathway is involved in TNFα-stimulated mPGES-1 expression is in line with reports on IL-β-stimulated A549 cells and LPS-stimulated macrophages.[172,199] The glucocorticoid dexamethasone, which is known to act through the suppression of NF- B and/or the transcription factor Activator Protein-1,[259] inhibited cytokine-induced mPGES-1 expression in gingival fibroblasts, further supporting a role for NF-κB in mPGES-1 regulation. Notably, it has been suggested that the inhibitory effects on mPGES-1 exhibited by anti-inflammatory natural products urolithin-A (from metabolised pomegranate) and curcumin (from the spice turmeric) are partly mediated through the inhibition of the JNK and NF-κB pathways.[195,260]

The transcription factor Egr-1 has been shown to be involved in the regulation of mPGES-1 expression in cell types including HeLa cells, murine osteoblastic cells and murine macrophages.[198-200] Although we found Egr-1 expressed in gingival fibroblasts, the Egr-1 stimulator phorbol-12-myristate-13-acetate (PMA)[261] did not increase mPGES-1 expression. The possible involvement of Egr-1 in cytokine-induced mPGES-1 expression in gingival fibroblasts therefore merits further study.

The mPGES-1 promoter contains regulatory elements including activator protein-1, glucocorticoid receptor (GR) and early growth response-1 (Egr-1) binding sites.[196,197] Moreover, the transcription factors Egr-1 and NF-κB have been shown to be involved in the regulation of mPGES-1 expression in different cell types including HeLa cells, pulmonary A549 cells, murine osteoblastic cells and murine macrophages.[172,198-200] In gingival fibroblasts, we have previously reported that the inflammatory mediators IL-1β and TNFα induce the expression of mPGES-1 in parallel with PGE_2 production and that the glucocorticoid dexamethasone inhibits the expression of mPGES-1.[157]

Regulation of mPGES-1 by components of the PGE_2 biosynthetic pathway (Study III)

In Studies III and IV we investigated the signal transduction pathways involved in the cytokine-induced mPGES-1 expression and PGE_2 production in gingival fibroblasts. Apart from signal transduction pathways, a co-ordinated induction of PGE_2 synthesis might also include inter-regulation between different components of the PGE_2 biosynthetic pathway. For this reason, we also investigated whether mPGES-1 and COX-2 expression was affected by the downstream prostaglandins or the upstream prostaglandin precursors. We found that the treatment of gingival fibroblasts with the end product PGE_2 or the downstream prostaglandin $PGF_{2\alpha}$ increased the expression of mPGES-1 and COX-2, suggesting a positive feedback loop in prostaglandin synthesis. In accordance with our results, positive feedback regulation of mPGES-1 by exogenous PGE_2 has been demonstrated in synovial fibroblasts,[262] although there are, to our knowledge, no published data demonstrating positive feedback by $PGF_{2\alpha}$.

The suggested anti-inflammatory prostaglandin 15-deoxy-Δ12,14-prostaglandin J_2 (15d-PGJ_2) reduced mPGES-1 expression in gingival fibroblasts. The inhibition of mPGES-1 expression by 15d-PGJ_2 has also been demonstrated in synovial fibroblasts, Chinese hamster ovary cells and rat chondrocytes.[159,263-265] Since 15d-PGJ_2 is the endogenous ligand of the transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ),[266] the effect on mPGES-1 expression may be mediated through PPAR-γ

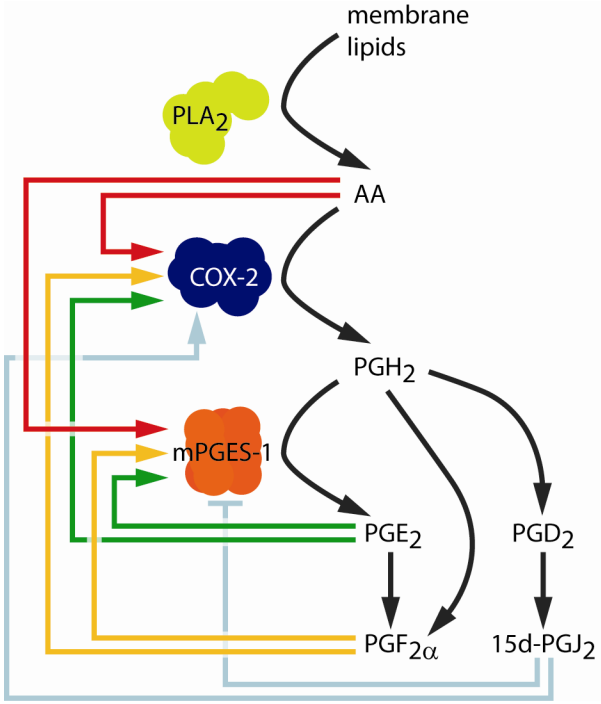

Figure 14. Schematic illustration of the results from Study III regarding regulation of mPGES-1 and COX-2 by components of the prostaglandin biosynthetic pathway in gingival fibroblasts. The arrows signify stimulation and the line with a T-shaped ending signifies inhibition of gene expression.
Abbreviations: 15d-PGJ_2, 15-deoxy-Δ12,14-prostaglandin J_2; AA, arachidonic acid; COX, cyclo-oxygenase; mPGES-1, microsomal prostaglandin E synthase-1; PLA_2, phospholipase A_2; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGH_2, prostaglandin H_2.

signalling. Notably, a PPAR-γ-independent pathway for the inhibition of mPGES-1 expression has been demonstrated in rat chondrocytes, where it was instead proposed that 15d-PGJ_2 inhibits the NF-κB pathway.[265] In contrast to its effect on mPGES-1, 15d-PGJ_2 increased COX-2 expression and PGE_2 production in gingival fibroblasts, further emphasising the un-co-ordinated regulation of mPGES-1 and COX-2.

Regarding the upstream precursors of PGE_2, we showed that the addition of exogenous AA, i.e. an increased amount of substrate, enhanced mPGES-1 and COX-2 expression in gingival fibroblasts. In accordance with this finding, the chemical inhibition of the enzyme PLA_2, which catalyses the formation of AA, reduced cytokine-induced mPGES-1 expression in the cells. These findings indicate that PLA_2 is involved in regulating mPGES-1 expression in gingival fibroblasts. However, the stimulatory effect of exogenous AA on the expression of mPGES-1 and COX-2 might also be mediated by an increased amount of PGE_2 and $PGF_{2\alpha}$ synthesis due to increased substrate availability. The effects of prostaglandins and prostaglandin precursors on mPGES-1 and COX-2 expression in cytokine-stimulated gingival fibroblasts are illustrated in Figure 14. The stimulatory effect of AA, PGE_2 and $PGF_{2\alpha}$ on mPGES-1 and COX-2 expression might be involved in causing a co-ordinated up-regulation of the enzymes of the PGE_2 biosynthetic pathway in inflammation. Furthermore, the positive feedback loop involving PGE_2 and $PGF_{2\alpha}$ could contribute to the self-perpetuation of the local inflammatory reaction.

Main findings

- The PGE synthases mPGES-1, mPGES-2 and cPGES, as well as COX-2, are expressed in inflamed gingival tissue from patients with periodontitis. In the gingival connective tissue, fibroblasts, endothelial cells, smooth muscle cells and inflammatory cells express PGE synthases.

- Cultured gingival fibroblasts, human airway smooth muscle (HASM) cells, human umbilical vein endothelial cells (HUVECs) and mast cells express mPGES-1, mPGES-2 and cPGES, as well as COX-2 and consequently produce PGE_2. The inflammatory cytokines TNFα and IL-1β stimulate the expression of mPGES-1 and COX-2 in gingival fibroblasts and HASM cells, accompanied by increased PGE_2 production. In HUVECs, TNFα increases COX-2 expression and PGE_2 production but not mPGES-1 expression. In mast cells, expression of PGE_2-synthesising enzymes and PGE_2 production are not affected by TNFα or IL-1β.

- Inhibition of mPGES-1 expression by siRNA does not affect the expression of mPGES-2 and cPGES, indicating that of the three PGE synthase isoforms, mPGES-1 is the most important enzyme in cytokine-induced PGE_2 synthesis in gingival fibroblasts.

- In gingival fibroblasts the up-regulation of mPGES-1 and COX-2 expression by TNFα involves the signal transduction pathways NF-κB and JNK, and their up-regulation by IL-1β involves NF-κB.

- In contrast to mPGES-1, cytokine-stimulated COX-2 expression is regulated by PKC, tyrosine kinase and p38 MAP kinase, which suggests a lack of co-regulation of these two enzymes in gingival fibroblasts.

- The prostaglandins PGE_2 and $PGF_{2\alpha}$ have a stimulatory effect on mPGES-1 and COX-2 expression, whereas 15d-PGJ_2 down-regulates mPGES-1 expression but up-regulates COX-2 expression.

Concluding remarks

The inflammatory mediator PGE_2 is involved in the pathogenesis of periodontitis. The terminal enzymes in PGE_2 biosynthesis, the PGE synthases, are currently in the focus of research and are regarded as possible therapeutic targets in chronic inflammatory conditions. We demonstrate that all three PGE synthases are expressed in gingival tissue from patients with periodontitis. Furthermore, we show that the isoenzyme mPGES-1 is the most essential of the PGE synthases in inflammation-induced PGE_2 production in gingival fibroblasts. This indicates mPGES-1 as an interesting object of research when it comes to understanding and possibly also helping to control chronic inflammation in periodontal tissue.

Our findings that the increased mPGES-1 expression in cytokine-stimulated gingival fibroblasts involves the signalling pathways JNK and NF-κB but not PKC, tyrosine kinase or p38 MAP kinase provide new insights into mPGES-1 regulation in gingival inflammation. We also show that the prostaglandins PGE_2 and $PGF_{2\alpha}$ may be involved in a positive feedback loop which enhances mPGES-1 expression, a mechanism which may contribute to the self-perpetuation of the local inflammatory reaction.

Our results suggest that the inflammation-induced production of PGE_2 by gingival fibroblasts is mediated by the increased expression of mPGES-1 and COX-2 and may possibly contribute to sustaining the chronic inflammation in periodontitis-affected gingival tissue. However, future studies investigating the effect of mPGES-1 inhibition *in vivo* and continuing to explore its regulation *in vitro* would be of great importance in order to further elucidate the role of the PGE synthases in periodontitis.

Acknowledgements

Six years after I registered as a PhD student at the Department of Dental Medicine, I am now ready to present my thesis. It has been a long journey, and a lot of people have helped me during these years. I would like to take the opportunity to thank some of them in particular.

Först och främst vill jag tacka min huvudhandledare **Tülay Yucel-Lindberg**. Du har ett outtröttligt driv framåt och ser till att varje motgång vänds i en framgång. Tack för att du har delat med dig av din kunskap både inom forskningsområdet och om vetenskaplig metodik. Tack också för att du har skapat en forskargrupp med en härlig stämning som främjar gott samarbete och fruktsamma vetenskapliga diskussioner.

Min bihandledare **Thomas Modéer** vill jag tacka för att du har lärt mig hur den akademiska världen fungerar, för att du har delat med dig av din insikt i den kliniska aspekten av vår forskning, för att du alltid ser den större bilden, och för att du har lärt mig att bara dra *lagom* stora växlar på mina resultat.

Ett stort tack till nuvarande och tidigare gruppmedlemmar i vår forskargrupp: **Anna Kats**, för att det är så skönt att ha någon bredvid sig som forskar på nästan samma sak som jag. Tack för alla samarbeten med texter, figurer, experiment och mycket annat. Tack också för att du är en så trevlig rumskamrat att nästan dela sittplats med! **Tomomi Kawakami**, thank you for contributing so much to our research and for teaching me a lot, both inside and outside of the lab. **Helena Domeij**, tack för att du introducerade mig till livet som doktorand, för glasspauser i solen och för att du lärde mig att märka in celler till FACSen. **Blanca Silva Lopez**, tack för all hjälp med snittning av biopsier, och för allt jobb du gjorde innan jag ens började, men som jag fortfarande har nytta av. Tack till min inte längre så nytillkomna rumskompis **Haleh Davanian**, för vetenskapliga diskussioner, för åsikter om högt och lågt, för kulturella insikter och för att du är så rolig att jobba med! **Pierre Georgsson** för hjälp med figurer och mycket annat, och för att du är med och skapar god stämning i gruppen. Tack till **Caroline Nguyen** för din hjälp med labbandet och för dina mycket noggranna anteckningar som jag fortfarande har nytta av. Tack också **Georgios Tsilingaridis**, **Elin Luttropp**, **Kaja Kraszewska**, **Cecilia Blomberg** och **Ying Ye** för trevlig stämning och gott samarbete.

Tack till **Marie-Louise Olsson** för att du vet allt som man behöver veta om metoder, kemikalier, labbrutiner och intranät, för att du gärna lånar ut reagens, skålar och plattor, samt för trevligt sällskap på tåget. Tack också till **Inger Carlsson** för all hjälp på labbet. Tack också till alla på **pedodontiavdelningen** för trevlig samvaro när vi har varit på kick-off och andra roligheter.

Tack till mina medförfattare: **Joakim Lundeberg**, för att jag fick komma och labba hos er på KTH, och för roliga och vetenskapliga diskussioner. **Johan Lindeberg**, för ett roligt och givande samarbete med stimulerande diskussioner (och för att du tackade mig så fint i din avhandling). Stort tack till **Hernàn Concha Quesada** för all din hjälp med FACSen, och för att du alltid ställer upp och fixar när man ringer! Till **Gareth Morgan**, tack för all din hjälp med mikroskopering, fotografering och framför allt tolkning av våra vävnadssnitt. Thanks also to our collaborators in Nottingham: **Alan Knox** and **Lisa Corbett** for sharing your knowledge and your HASM cells. Till våra samarbetspartners inom KI: **Per-Johan**

Jakobsson och **Marina Korotkova**, tack för att vi fick möjligheten att arbeta med era knockade möss och för att jag fått del av er stora kunskap om PGE syntaserna. **Gunnar Nilsson**, tack för hjälp med mastceller och manuskriptläsning. Tack till **Idil Burt** och **Leonardo Pino** för hjälp med de gingivala biopsierna. Tack också till **Jan Bergström** för att du läste avhandlingens introduktion med parodontolog-glasögon och till **Rachael Sugars** för att du läste och förbättrade mitt abstract.

Sometimes, when life as a PhD student gets you down (and also when it doesn't and you have time for long fika breaks), it's good to know that you are not alone. Thank you to my fellow PhD students and postdocs (former and present) at the department: **Anna P**, tack för allt roligt vi hade medan vi delade rum, och för allt du har lärt mig om CAD/CAM och tandteknikeri. **Peggy**, tack för luncher, skjutsar och diskussioner om allt mellan himmel och jord. **Lena**, tack för luncher, fikapauser och allmänt trevligt umgänge. **Ai**, tack för att du var en så trevlig rumskamrat, och för stimulerande diskussioner om svenska språket. Thanks also to **Maha**, **Hero**, **Fawad**, **Abier**, **Yuko**, **Anna-Kari**, **Nikos**, **Lars F**, **Aron**, **Greg**, **Anna-Karin**, **Staffan, Mei Ling** and **everyone else** who I probably forgot, for banter and good times at the department. Tack också till **Daniel Klevebring**, numera teknologie doktor, för inspiration till avhandlingens layout.

Jag vill även skicka ett litet tack till min gymnasielärare i biologi, **Bosse Milton**, som inspirerade mig till att fortsätta på den (molekylär)biologiska banan. Jag hade tänkt tacka dig när jag fick Nobelpriset, men det här är nog det närmaste jag kommer...

Utanför den akademiska världen finns det tydligen något annat. Den riktiga världen. Där finns många fina människor som jag inte klarar mig utan: Tack till de Fantastiska Flickorna **Åsa**, **Anna**, **Mia**, **Petra**, **Basia**, **Martina** och de (nästan) lika fantastiska **pojkarna** som hör till. Våra middagar på stan har räddat mig många gånger! Tack också till **Camilla** för att du är så bra att prata med om allt möjligt.

Till **Linda**, **Sofie**, **Nisse**, **Qaryn**, **Lindizz**, **Kurta**, **Magnus P**, **Qlof**, **Ankan, Sara**, **Perikles** och alla andra härliga **spexare** i Kårspexet på KTH: Tack för att ni har hjälpt mig fylla min fritid med annat än forskning! Exempelvis orkesterrepetitioner, ordvitsande, föreställningar på Stockholms stora teatrar, PR-bludder, dans, sång och mycket annat som nog inte passar att nämna i denna text.

Tack till mina kära **föräldrar** som alltid stöttar mig och som gärna tar hand om barnbarnen så att jag och Pär får tid för annat (exempelvis för att skriva avhandling). Tack också för det naturvetenskapliga tankesätt som jag har fått med modersmjölken och som har gjort att jag har tagit mig ända fram till en doktorsexamen. Tack också till mina syskon **Sara** och **Johan** för att vi har så roligt tillsammans!

Tack till mina fina små pojkar **Vidar** och **Linus**, för att ni ser till att jag förblir fast förankrad i vardagslivet, och för att ni är så underbara!

Och tillslut ett stort tack till min man **Pär**, min klippa som har stöttat mig genom hela doktorandtiden. Detta hade aldrig gått utan dig. Ett särskilt tack för att du har skött allt (jag menar allt) hemma under den sista tiden av avhandlingsarbetet. Jag ser fram emot många fina år med dig framöver!

To **everyone else** who I forgot but who deserves to be thanked: Thank you! (And sorry I forgot you.)

My work was financially supported by grants from the Swedish Research Council, the Swedish Patent Revenue Fund and Karolinska Institutet.

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