Inhibitory Effects of Natural Flavones on Mast Cell and Keratinocyte Activation

A Dissertation

Submitted by

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In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Pharmacology and Experimental Therapeutics

TUFTS UNIVERSITY

Sackler School of Graduate Biomedical Sciences

December, 2013

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ABSTRACT

Mast cells are immune cells derived from the bone marrow that can rapidly release numerous preformed and *de novo*-synthesized mediators like tumor necrosis factor (TNF), thereby participating in various immune and inflammatory conditions. Recent evidence indicates increased infiltration of mast cells in psoriatic lesional skin. Psoriasis (Ps) is an autoimmune disease characterized by keratinocyte hyperproliferation and chronic inflammation, with increased expression of TNF and vascular endothelial growth factor (VEGF). Keratinocytes can interact with dermal mast cells and participate in the pathogenesis of Ps. Anti-TNF biological agents are more effective than traditional treatments for Ps, but they are associated with increased risk of infections and blood malignancies. In addition, these agents target inflammatory mediators after they are released, and do not address keratinocyte hyperproliferation or activation. Thus, the need for safe and effective treatments for Ps is still of major importance.

Flavones are plant-derived natural compounds with potent antioxidant and anti-inflammatory actions. Flavones can also inhibit mast cell mediator release, but their precise mechanism of action on human mast cells is not well understood, and their effect on human keratinocytes has not been fully explored. In the present study, we investigated effects of two flavones, luteolin and its structural analogue 3',4',5,7-tetramethoxyluteolin (methoxyluteolin), on mediator release from human mast cells and keratinocytes. We hypothesized that the two flavones inhibit activation and proliferation of mast cells and keratinocytes, thereby providing novel benefits in the treatment for Ps.

We show that luteolin and methoxyluteolin are more potent than the only clinically available "mast cell stabilizer" disodium cromoglycate (cromolyn), and block inflammatory mediator production in human mast cells and keratinocytes. This inhibitory effect is mediated by blocking intracellular calcium elevation, reducing mitochondrial translocation, and decreasing NF-κB activation at both the transcriptional and translational levels. The two flavones also decrease keratinocyte proliferation without affecting intracellular energy production. Hence, luteolin and methoxyluteolin are promising candidates for development of safer and more effective alternative therapies for the treatment of skin diseases such as Ps, as well as other inflammatory conditions.

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ACKNOWLEDGMENTS

I am deeply grateful to the many people who, during the past four years, have contributed to the successful completion of my graduate studies at Tufts. In particular, I am greatly indebted to my thesis advisor, Dr. Theoharides, for his guidance and continued support, and my thesis committee members, Dr. John J. Castellot (Chair), Dr. Louis Shuster and Dr. Angelo Azzi, for being terrific mentors, as well as my outside examiner, Dr. Aristidis Veves, for being an incredibly valuable resource.

I would like to thank Dr. Richard Shader, who gave me a precious interview opportunity at Tufts four years ago. I am also deeply grateful to all PPET faculty for their continued guidance. I would like to express my sincere gratitude to Dr. David Greenblatt and my fellow student Lei Cao for their great help with my LCMS experiments. I would like to thank Dr. A. Slominski (University of Tennessee, Menphis, TN) for providing human HaCaT keratinocytes. I am also grateful to Dr. A Kirshenbaum (NIH, Bethesda, MD) for providing human LAD2 mast cells.

Special thanks to my laboratory colleagues, who have always been by my side for advice, collaboration and laughter: Bodi Zhang, Magdalini Vasiadi, Arti Patel, Shahrzad Asadi, Nikolaos Sismanopoulos, Danae-Anastasia Delivanis, Alexandra Miniati, Anastasia Therianou, Michail Alevizos, Anna Karagkouni and Smaro Panagiotidou. Many thanks to our department staff as well, who always helped me with everyday tasks without hesitation.

I also wish to express my deep gratitude to my fellow students and friends, especially Najla Fiaturi, Bai Huijun, Luo Chi, Yang Hailing, Sheng Jinghao, Jiang Yuxiang, Qiu Jin, Chen Weijun, Huang Shuoxi. Lastly, I would like to thank my parents and my husband for their endless love and support, and for encouraging me to pursue my passion for science.

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Caco-2	Human intestinal epithelial cell line
CCL2	C-C motif chemokine ligand 2
COX-2	Cyclooxygenase 2
CRH	Corticotropin-releasing hormone
Cromolyn	Disodium cromoglycate
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
EGCG	Epigallocatechin gallate
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FcεRI	High affinity IgE receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hours
HaCaT	Human keratinocyte cell line
hCBMCs	Human umbilical cord blood-derived cultured mast cells
HepG2	Human hepatocyte cell line
HKGS	Human keratinocyte growth supplement
HMC-1	Human mast cell-1
IFN-α	Interferon-α
IgE	Immunoglobulin E
IL	Interleukin
iNOS	Inducible nitric oxide synthase

IP3	Inositol 1,4,5-triphosphate
LCMS	Liquid chromatography-mass spectrometry
LPS	Lipopolysaccharides
LTB_4	Leukotriene B_4
LTs	Leukotrienes
Lut	Luteolin
mBMMC	Mouse bone marrow-derived cultured mast cells
Methlut	3',4',5,7-tetramethoxyluteolin
NF-κB	Nuclear factor-kappa B
NFKB1	Gene encoding NF-κB p50 subunit
NGF	Nerve growth factor
NHEKs	Normal human epidermal keratinocytes
NK	Neurokinin
NT	Neurotensin
PAF	Platelet activating factor
PGD_2	Prostaglandin D_2
PI3K	Phosphoinositide 3-kinases
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
Ps	Psoriasis
PUVA	Psoralen plus ultraviolet A phototherapy
qRT-PCR	Quantitative real time-polymerase chain reaction

RELA	Gene encoding NF-κB p65 subunit
rhSCF	Recombinant human stem cell factor
SNARE	Soluble NSF attachment protein receptor
SOCE	Store-operated calcium entry
SP	Substance P
TGFβ	Transforming growth factor β
TNF	Tumor necrosis factor-α
TNFAIP3	TNF-α-induced protein 3
UGT	Uridine diphosphate glucuronosyltransferase
UVA	Ultraviolet A
VAMP-8	Vesicle-associated membrane protein 8
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
WSQ	Water-soluble quercetin

Chapter 1. Introduction and Objectives

Mast cell development and maturation

Mast cells are immune cells derived from hematopoietic progenitor cells originating in the bone marrow[1, 2]. Committed bone marrow mast cell progenitors are released into the bloodstream and subsequently migrate into tissues throughout the body, where they become differentiated and mature under the influence of tissue-specific growth factors and cytokines[1, 2]. In particular, mast cell development depends on stem cell factor (SCF)-induced activation of the tyrosine kinase c-kit receptors present on their plasma membrane[3, 4]. As a result, human mast cells in culture require SCF for their survival and growth. In the continued presence of SCF, mature mast cells are long-lived in tissues. Apart from SCF, interleukin (IL)-4 is another important regulator of mast cell development and function. IL-4, together with SCF, enhances proliferation rate and mediator release of human intestinal mast cells[5], in addition to nerve growth factor (NGF)[6].

Mast cells typically reside in tissues interfacing with the external environment, including the skin, respiratory tract and intestines, as well as near blood vessels[7]. A striking feature of mature, especially connective tissue mast cells, is that their cytoplasm is filled with numerous granules, where preformed mediators are stored[8]. Paul Ehrlich in 1878 first identified mast cells and speculated these cells existed to feed the surrounding tissue with their dense granules; hence he named them mast cells (from the Greek word for Breast). He was later awarded the Nobel Prize in Physiology or Medicine in 1908.

Upon activation, mast cells rapidly release preformed mediators through degranulation, as well as the delayed secretion of newly synthesized cytokines, chemokines and growth factors[8]. Increasing evidence indicates that mast cells participate in allergy, innate and acquired immunity[9-11], autoimmunity[12], and inflammation[7, 13, 14].

Mast cell mediators

Mast cell granules contain various preformed mediators, including biogenic amines (histamine in human mast cells, serotonin in rodent mast cells), enzymes (β-hexosaminidase, tryptase and chymase), proteoglycans (heparin, chondroitin sulfate and hyaluronic acid), as well as the preformed cytokine tumor necrosis factor (TNF)[15]. Upon stimulation, mast cells release these preformed mediators through rapid degranulation within 5-30 min[16]. Lipid mediators, such as leukotriene B_4 (LTB_4), LTC_4, prostaglandin D_2 (PGD_2) and platelet activating factor (PAF) are synthesized during mast cell activation from arachidonic acid liberated by the action of phospholipases[8]. Mast cell activation also induces *de novo* synthesis and secretion (6-24 hours later) of various cytokines and chemokines[17], including interferon-α (IFN-α), interleukin (IL)-1β, IL-3, IL-4, IL-5, IL-6, IL-13, TNF, IL-8 (or CXCL8) and chemokine (C-C motif) ligand 2 (CCL2), as well as growth factors such as SCF, granulocyte-macrophage colony-stimulating factor (GM-CSF), nerve growth factor (NGF) and vascular endothelial growth factor (VEGF), some of which can be released selectively without degranulation[18]. It is important to note that mast cells are heterogeneous, and different populations with varied mediator profiles are observed in distinct tissues, for instance, all mast cells contain tryptase, but only connective tissue mast cells also contain chymase[19]. Mast cell characteristics of different

populations are not static either, since they change phenotype in the context of inflammation or infection[20].

Allergic and non-allergic triggers of mast cells

Mast cells are necessary for the development of allergic reactions mediated by immunoglobulin E (IgE)[21-24]. Mast cells express the high affinity IgE receptor (FcεRI) on the cell surface[25, 26]. Upon exposure to exogenous antigens, B cells produce IgE that binds to the high affinity FcεRI receptors on mast cell surface, a process called mast cell priming[27]. Re-exposure to the same antigen or other macromolecules leads to aggregation and cross-linking of FcεRI receptors by the IgE-bound antigen, resulting in massive degranulation by compound exocytosis to rapidly release preformed mediators[28]. The degranulation process involves translocation of cytoplasmic granules along microtubules towards the plasma membrane followed by the calcium-dependent assembly of "docking proteins" that belong to the same family as the Soluble NSF Attachment Proteins (SNAREs)[29], resulting in membrane fusion and granule exocytosis[16].

Mature mast cells are distributed throughout the body including the brain, whilst IgE is unable to cross the blood-brain barrier. Moreover, mast cells can induce inflammatory and immune responses without causing massive degranulation and anaphylactic shock, as mediated by IgE[18], a process called "selective" or "differential" release[30]. For example, mast cells can release specific mediators such as serotonin without histamine[30]. LPS triggers *de novo* synthesis and release of TNF from human mast cells without causing degranulation[31]. IL-1 stimulates human mast cells to selectively

release IL-6 without degranulation through small vesicles (40–80 nm) unrelated to the secretory granules (800–1000 nm)[32]. VEGF is also released from normal human cultured mast cells selectively in response to corticotrophin-releasing hormone (CRH)[33]. In fact, in addition to IgE, mast cells can be stimulated by various non-allergic triggers, such as bacterial or viral antigens (lipopolysaccharides (LPS), double-stranded RNA), complement peptides (C3a and C5a), mitochondrial components, cytokines, hormones (CRH), as well as chemical agents (compound 48/80 and morphine)[7, 18]. More importantly, skin mast cells are located close to sensory nerve endings and can be triggered by neuropeptides[34, 35], such as neurotensin (NT)[36], NGF [37] and substance P (SP)[38], which can be released from dermal nerve endings. Unlike IgE-mediated activation, peptide- and drug (such as SP and compound 48/80)-induced mast cell activation occurs through different G-protein coupled receptors[39, 40], and also receptor-independent mechanisms through direct or indirect activation of G proteins[41-43].

Mast cells in inflammation

Increasing evidence indicates the critical involvement of mast cells in a number of systemic and brain inflammatory diseases, including asthma, autism, multiple sclerosis, interstitial systitis, obesity and psoriasis (Ps)[7, 44, 45]. Mast cell-derived histamine and PGD_2 induce bronchoconstriction and mucus secretion, contributing to asthma[46]. Mast cell-derived histamine also induces matrix metalloproteinase-9 expression in keratinocytes and promotes collagen type IV degradation in the basement membrane[47]. Mast cells uniquely store preformed TNF[31], which is rapidly released upon stimulation and influences T cell recruitment and activation[48]. Mast cell-derived CCL2 and IL-8

enhance recruitment of immune cells to the site of inflammation[49]. Mast cell-derived VEGF increases local vascular permeability and promotes angiogenesis[50]. Stimulated live mast cells secrete mitochondrial components, which may act as "autopathogens" contributing to the pathogenesis of inflammatory diseases[45, 51]. In addition, activated mast cells release stable heparin-based particles or granules containing TNF and other proteins, which are able to rapidly enter lymphatic vessels and travel to remote draining lymph nodes[52]. These mast cell-derived particles effectively transfer signals from peripheral sites to secondary lymphoid tissues, facilitating the development of the adaptive immune response. More interestingly, synthetic mast cell granules, which consist of a carbohydrate backbone with encapsulated TNF, have been tested as an adjuvant in mice and shown to enhance adaptive immune responses in draining lymph nodes[53]. Based on their wide tissue distribution and their ability to rapidly release multiple mediators, mast cells actively interact with other cell types in their surrounding environment, and therefore play an important role in virtually every tissue, through participation in the induction and/or propagation of various immune and inflammatory responses.

Mechanisms involved in mast cell mediator release

Elevation of intracellular calcium ions

In mast cells, stimulation with antigen or other triggers leads to rapid elevation of cytoplasmic calcium, which is an essential signal for degranulation, generation of lipid mediators such as LTs and PGD_2, as well as production of cytokines and chemokines[26]. Trigger-stimulated calcium elevation is initiated by activation of phosphoinositide 3-kinases (PI3K) and phospholipase C (PLC) $\gamma 1$ and $\gamma 2$[54], which hydrolyze

phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane to produce inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG)[55]. DAG remains on the plasma membrane and activates protein kinase C (PKC) isoforms, which in turn activate other cytosolic proteins and contribute to granule exocytosis[56]. IP_3 is released into the cytoplasm and activates IP_3 receptors on the smooth endoplasmic reticulum (ER), which opens calcium channels on the ER and mobilizes stored calcium ions from the ER into the cytosol[55]. Depletion of intracellular calcium stores triggers a secondary, more pronounced, influx of extracellular calcium through the corresponding calcium channels on the plasma membrane[56], which is sustained by PI3K through amplification and maintenance of PLCγ activity[57]. This calcium influx process is commonly referred to as store-operated calcium entry (SOCE), which was originally demonstrated by Putney's group[58]. The influx of calcium replenishes intracellular calcium stores in the ER via an ATP-dependent calcium ATPase pump, and maintains an elevated cytosolic calcium level that results in mast cell activation[55].

Fission and translocation of mitochondria

Degranulation of mast cells requires metabolic energy[59]. Mitochondria are the primary sources of energy production in eukaryotic cells and also have the ability to buffer calcium locally[60]. Moreover, mitochondria are dynamic organelles that participate in many complicated cell functions through morphological and localization changes[61]. Increasing evidence indicates the importance of mitochondria dynamics in immune cell regulation. For instance, mitochondrial translocation is required for T cell "immune synapse" formation and sustainable calcium influx[62]. On the other hand, local

intracellular calcium changes can also regulate mitochondrial dynamics and their subcellular localization[63].

Previously, we had shown that in human mast cells, SP-induced preformed TNF secretion requires intracellular calcium increase, high mitochondrial energy consumption, as well as mitochondrial translocation to the cell surface[15]. Mitochondria may translocate close to the secretory granules in order to provide energy locally for the granules to fuse with the plasma membrane and undergo exocytosis as shown for lymphocyte chemotaxis[64]. Mitochondrial translocation may also be needed to maintain optimal local calcium levels necessary for granule exocytosis[59], most likely for the calcium dependent proteins involved in degranulation, such as the SNARE proteins and the Vesicle-Associated Membrane Protein 8 (VAMP-8)[65].

Activation of the nuclear factor kappa B (NF-κB)

The nuclear transcription factor NF-κB plays a pivotal role in the regulation of many cellular responses, such as inflammation and proliferation[66]. NF-κB is initially present in the cytoplasm as a heterodimer consisting of its p50 and p65 subunits. Upon stimulation, NF-κB is phosphorylated and translocates into the nucleus, where it binds to the promoter region of a number of target genes[66]. Previous evidence indicates that elevation of intracellular calcium ions activates protein kinase C, followed by induction of NF-κB that subsequently induces production of TNF, IL-6 and IL-8 in rat RBL-2H3 mast cells and human mast cells clone-1(HMC-1)[67, 68]. Furthermore, NF-κB activation leads to increased expression of a number of inflammatory molecules, including

cyclooxygenases (COX-2) and inducible nitric oxide synthase (iNOS) in both rat and human mast cells[69, 70].

Keratinocytes

The skin is the largest organ in the body and acts as a physical barrier to protect against harmful environmental and infectious agents. The skin consists of two layers, the outer epidermis and the underlying dermis. Keratinocytes are the major cell type residing in the epidermis. They are fully immunocompetent to release inflammatory mediators such as IL-1, IL-6, IL-8, TNF and VEGF, in response to immune and endocrine triggers[71-73]. TNF is a potent trigger of keratinocyte activation and further induces TNF secretion from keratinocytes in an autocrine fashion[74]. Keratinocytes express neurokinin (NK) 1 receptors and can be stimulated by SP [75] to release IL-1β[76]. Keratinocytes are involved in the pathogenesis of psoriasis (Ps), through their hyperproliferation and associated chronic inflammation[77-80].

Interactions between keratinocytes and skin mast cells

The dermis layer of the skin contains blood and lymphatic vessels, nerve endings, sebaceous and sweat glands, as well as mast cells. Dermal mast cells are often positioned closely to sensory nerve endings, blood vessels and hair follicles. As a result, mast cells are believed to play a part in regulating vascular permeability and angiogenesis in the skin[81], as well as host defense against infections[10]. Additionally, mast cells and keratinocytes can interact with each other by means of secretion of soluble mediators.

Keratinocytes have been shown to secrete SCF that acts as both a chemoattractant and a regulator of mast cell growth and maturation[82, 83]. Moreover, culture supernatants from differentiating human keratinocytes (at day 11 of culture) increased histamine and tryptase content in human HMC-1 mast cells, which was independent of SCF[84]. Activated keratinocytes could also produce IL-1β, which triggers mast cells to rapidly release preformed TNF. TNF could further act on keratinocytes to release a number of other inflammatory mediators.

Conversely, mast cell-derived histamine and serotonin induces keratinocyte proliferation in mice[85]. In addition, keratinocyte proliferation is accelerated by platelet-activating factor (PAF), which is also secreted from mast cells[86]. Mast cell-derived histamine increases NGF production from keratinocytes; NGF, in turn, stimulates histamine release from mast cells, creating a positive feedback loop[87]. Mast cells are the only cell type that store preformed TNF, which is rapidly released during mast cell degranulation that can subsequently stimulate keratinocytes to secrete IL-33, which is one of the newest members of the IL-1 family of inflammatory cytokines[88]. IL-33 is released rapidly after allergen challenge in asthma patients[89], can mediate IgE-induced anaphylaxis in mice[90]. IL-33 induces release of IL-6 from mouse bone marrow-derived cultured mast cells (mBMMC)[91], and IL-8 from human umbilical cord blood-derived cultured mast cells (hCBMCs)[92]. Previously, we showed that IL-33 augments SP-stimulated VEGF release from human mast cells; IL-33 gene expression is also increased in lesional skin of Ps patients[93].

Psoriasis

Ps is an autoimmune skin disease affecting approximately 2-3% of the world's population. The prevalence of Ps varies from 0.73 to 2.9% in Europe and from 0.7 to 2.6% in the United States. By contrast, the prevalence of Ps observed in Asian countries varies from no cases detected to 0.5% of the population. Ps is more common in adults, and more frequent in countries further away from the equator. With typical lifetime duration of more than 30 years, the combined cost of Ps has a major impact on health care[80]. Ps is characterized by keratinocyte hyperproliferation, T cell and neutrophil infiltration, increased skin capillaries and chronic inflammation[78]. Ps can also be triggered or exacerbated by acute stress[94-97]. Neuropeptides[98], especially SP[99], are involved in the pathogenesis of Ps. SP can activate mast cells[100], and induce adhesion molecules on endothelial cells, resulting in infiltration of other immune cells such as neutrophils and eosinophils[101].

A number of cytokines, chemokines and transcription factors have been implicated in the pathogenesis of Ps. TNF is a key cytokine involved in the initiation and progression of Ps [102] and is produced by a number of cell types, including activated keratinocytes, T cells and mast cells[80]. TNF can trigger keratinocytes to produce IL-1[103], which stimulates both keratinocytes and mast cells to release IL-6[32]. IL-6 then exerts autocrine actions and stimulates proliferation of cultured human keratinocytes[104]. TNF stimulates keratinocytes to release VEGF, which leads to dysregulated angiogenesis that contributes to the development of Ps-like dermatitis[72, 105]. In addition, TNF triggers

activation of nuclear factor-kappa B (NF-κB), a transcription factor involved in inflammatory mediator production, which is upregulated in Ps lesional skin[106].

Recent evidence also indicates increased mast cell infiltration and activation in lesional skin from Ps patients as compared to normal skin[77, 77, 79, 97]. Interestingly, SP-positive nerve fibers are more dense in psoriatic lesions and are localized close to dermal mast cells[97, 107, 107-109]. SP can stimulate mast cells [110, 111] and contributes to inflammation[112, 113]. SP-positive nerve fibers and mast cell contacts are also increased by acute stress in mice[114], leading to dermal mast cell degranulation[95, 110, 115].

Mast cells are also important for maturation of Th17 cells, which are recognized as key cells in autoimmune disorders by releasing IL-17 and IL-22[116]. For instance, mast cells in the presence of IL-6 and transforming growth factor β (TGFβ) are necessary for the production of Th-17 cells[117], while TNF and vasoactive intestinal peptide (VIP) drive IL-6-independent Th17 cell maturation[117-119]. A previous finding suggests that mast cells may play a significant role in immunity by releasing IL-17[120], which is a "driver" cytokine that induces inflammation in Ps patients [121] and stimulates IL-8 production in normal human epidermal keratinocytes (NHEKs)[122]. Moreover, a recent genome-wide association study has found considerable overlapping single nucleotide polymorphisms between Ps and asthma, where mast cell activation is critically involved[123]. Mast cells may, therefore, be involved in the pathogenesis of Ps and other inflammatory skin diseases.

Current treatments for psoriasis

Currently, there is no cure for Ps, but various treatments are available to help control the symptoms. Topical treatments are typically used for mild Ps, including creams and ointments containing corticosteroids, retinoids and vitamin D_3 analogues[80, 124]. Phototherapy is used for moderate Ps, such as psoralen plus ultraviolet A phototherapy (PUVA), which combines the oral or topical administration of psoralen with exposure to ultraviolet A (UVA) light that can inhibit skin cell proliferation[125]. Severe Ps that is resistant to topical treatments and phototherapy is treated with systemic agents, including the two immunosuppressant drugs methotrexate and cyclosporine, as well as retinoids[80, 124]. However, these treatments are associated with unwanted adverse effects including nausea, headache, fatigue, burning, and itching, as well as increased risk of birth defects[126].

As the role of TNF in Ps pathogenesis has become more recognized, anti-TNF biologic agents, such as etanercept, have been developed and shown to be more effective than traditional treatments for Ps. Unfortunately, these anti-TNF agents are also associated with increased risk of infections, and more recently blood malignancies[127, 128]. Other biologic agents have been introduced targeting IL-12, IL-17 and IL-23[129, 130], but the long-term efficacy data remain unknown[131]. Therefore, the need for safe and effective long-term treatments for Ps is still of major importance.

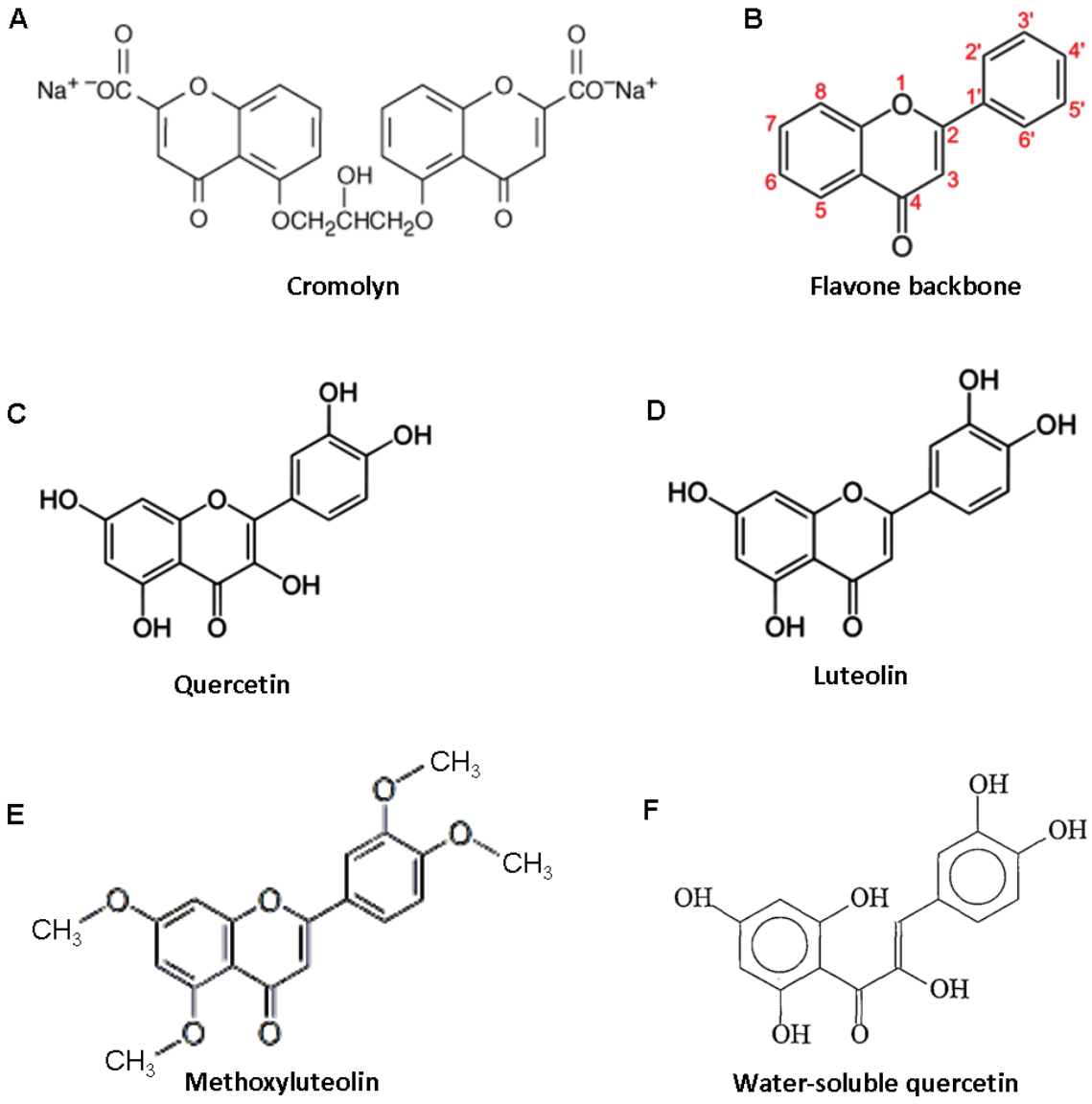
Currently available mast cell secretion inhibitors

Due to the critical involvement of mast cells in Ps and other inflammatory diseases, it would be advantageous to inhibit secretion of inflammatory mediators from mast cells or other immune cells. There appear to be some "innate" inhibitors of mast cell secretion. Chondroitin sulfate and heparin, the major constituents of mast cell granules, inhibit human mast cell mediator release[132]. Nitric oxide blocks FcεRI-mediated mast cell cytokine secretion[133]. In addition, the anti-inflammatory cytokine IL-10, which is also secreted by mast cells themselves, has been recently shown to inhibit IgE-triggered histamine and TNF release from hCBMCs[134]. However, none of these molecules has been developed for clinical use.

Currently, there are no *effective* mast cell inhibitors available in the clinics. The antihistamine ketotifen, which partially inhibits mast cells[135], is only available for allergic conjunctivitis in the USA, while the antihistamine rupatadine that can inhibit cytokine secretion from human mast cells [136] is not available in the USA. The only clinically available "mast cell stabilizer" cromolyn (Fig. 1.1 A) inhibits rat peritoneal mast cell histamine secretion[137], but could not inhibit rat mucosal mast cells[138, 139]. Moreover, cromolyn is a very weak inhibitor of human mast cells[140, 141]. In fact, 1000 μM cromolyn was required for inhibition of histamine release from human tonsillar mast cells[142], and was ineffective in inhibiting human lung and intestinal mast cells[143]. In addition, intestinal absorption of cromolyn is severely limited (≤1%), and several weeks of therapy may be needed before any clinical benefits are seen[144]. Several studies even reported development of contact dermatitis from cromolyn-containing eyedrops[145, 146]. As a result,

there is urgent need for developing effective inhibitors of human mast cell mediator release.

Figure 1.1. Structures of cromolyn and flavonoids.



Flavonoids

In search for potential mast cells inhibitors, we recognized that the structure of cromolyn is quite similar to the backbone of flavones (Fig. 1.1 B). Flavones belong to a group of naturally occurring compounds, collectively called flavonoids, which occur in a number of plants and fruits such as onions, tea, olives, corns and citrus fruits. Flavonoids have been suggested to have a wide spectrum of potential health benefits, including antioxidant, anti-inflammatory, antiallergic, anticarcinogenic, antiobesity, antidiabetic, and hepato- and gastro-protective effects[147-150].

More importantly, several flavonoids can inhibit histamine release from murine mast cells[151, 152], as well as IL-6 and TNF release from bone marrow-derived cultured murine mast cells (mBMMCs) and rat peritoneal mast cells[153]. The flavonol quercetin (Fig. 1.1 C) inhibits the release of histamine, LTs and PGD_2 from human mast cells in response to IgE[154]. Recently luteolin (Fig. 1.1 D), a flavone structurally related to quercetin, has been shown to inhibit IgE-mediated histamine and IL-6 release from mBMMCs and rat peritoneal mast cells[153]. However, the precise mechanisms of action of luteolin on human mast cells are not fully understood.

Subgroups of flavonoids

Flavonoids are plant secondary metabolites and are usually found as glycosides (i.e., with attached sugars). Occasionally, flavonoids also occur in plants as aglycones[155]. The common backbone of flavonoids contains two aromatic rings linked through three

carbons (Fig. 1.1 B). Flavonoids can be further classified into six major subgroups, based on their chemical structure: flavones (e.g., diosmetin and luteolin), flavonols (e.g., kaempferol and quercetin), flavanones (e.g., hesperetin and naringenin), catechins (e.g., epicatechin and gallocatechin), anthocyanidins (e.g., cyanidin) and isoflavones (e.g., genistein)[147, 156].

Absorption, metabolism and toxicity of flavonoids

The absorption of flavonoids and their metabolism have been studied *in vitro* using human intestinal epithelial cells (Caco-2) and hepatocytes (HepG2) because the gut and the liver are two major organs for flavonoid absorption and metabolism[157]. Flavonoid aglycones could get into cells by passive penetration through the plasma membrane due to their planar structures[158]. In addition, flavonoids can utilize several transporters present on the cell surface. For example, quercetin glucosides compete with glucose for transport by the intestinal Na-dependent glucose transporter 1[159]. In fact, glycosyl conjugation of quercetin with specific sugar moieties can effectively enhance oral bioavailability of quercetin in mice[160]. Absorption of certain flavonoid also depends on their lipophilicity and specific interactions with efflux transporters, such as P-glycoprotein and breast cancer resistance protein[161, 162].

Flavonoids are mainly metabolized by phase II enzymes in the gut and the liver before being released into systemic circulation[163, 164]. Due to its poly-phenol structure, luteolin is a good substrate for uridine diphosphate glucuronosyltransferases (UGTs) and sulfotransferases, and undergoes extensive glucuronidation and sulfation in rats and

humans[165]. After intravenous administration in rats, luteolin is also metabolized by catechol-*O*-methyltransferase to yield two methylated metabolites, which have their own pharmacological effects[166].

Due to extensive metabolism, plasma levels of quercetin and luteolin aglycones are normally low. Several studies have shown that peak plasma concentrations of quercetin and luteolin in humans on a normal diet do not exceed 10 μM[167]. The relative urinary excretion of flavonoids is less than 10% of the ingested dose, depending on the flavonoid structure[168, 169], and elimination half-lives range from 6 to 28 hours[170]. It has been shown that quercetin and luteolin are bioavailable from foods such as onions, tea and citrus fruits[171]. Quercetin has also been shown to traverse across the blood-brain barrier using *in situ* rat models[172]. The estimated daily consumption of flavonols and flavones varies between 27-100 mg/day in a balanced diet[173, 174]. Repeated intake of food supplements containing high doses of flavonoids (1 g quercetin per day) results in increased plasma concentrations reaching about 5 μM of the nonconjugated form[169]; this translates to about 12% oral absorption assuming equal distribution in an 80 L single-compartment model.

Flavonoids are usually considered as non-toxic, and administration of high doses of quercetin and luteolin showed no acute toxicity in various animals[175]. A recent study showed that a formulation of quercetin and luteolin were well tolerated in children[176]. Luteolin can even protect against chemically-induced hepatotoxicity [177] and nephrotoxicity[178], a common consequence of many therapeutic drugs.

Biological activities of flavonoids

Epidermiological studies have shown that consumption of flavonoid-rich foods reduces risk of cardiovascular disease, diabetes and certain types of cancer[147]. Due to the oxidative stress-related pathology of these diseases, the strong antioxidant property of flavonoids was initially thought to be the main mechanism of action, especially when considerng their polyphenolic structures[147, 179]. Some flavonoids, such as quercetin, can induce the expression of glutathione S-transferase and increase its activity[180]. Additionally, flavonoids have been shown to chelate some transition metal ions responsible for the production of reactive oxygen species[147].

Increasing evidence from *in vitro* and *in vivo* studies suggests that flavonoids also directly interfere with signaling cascades involved in inflammatory and immune responses[167]. A number of studies have shown inhibitory actions of flavonoids on cytokine production and activation of B cells, T cells and neutrophils[147]. Luteolin has been shown to inhibit inflammatory cytokine release in macrophages by blocking NF-κB activation[181, 182]. Luteolin can also inhibit proinflammatory enzymes like COX-2, iNOS and lipoxygenases[183].

Flavonoids also have antimicrobial properties. Quercetin can inhibit different stages in the replication cycle of viruses[184]. Luteolin has shown actions against several types of parasites[185]. Additionally, flavonoids have been shown to decrease proliferation of different cancer cells and inhibit angiogenesis[147]. Furthermore, luteolin can sensitize

cancer cells to anticancer reagents by suppressing cell survival pathways and stimulating apoptosis pathways[186].

More importantly, flavonoids regulate mast cell activation. Quercetin was first shown to inhibit histamine release from rat mast cells in 1977[187]. Quercetin, kaempferol and myricetin have been shown to decrease TNF, IL-6 and IL-8 release from hCBMCs by inhibiting protein kinase C theta[188]. Quercetin and luteolin also inhibit cytokine release from HMC-1 mast cells by blocking NF-κB activation[70, 189]. Moreover, luteolin reduces thimerosal-induced VEGF release from LAD2 mast cells[190]. However, the precise mechanism of action of luteolin on human mast cells are not fully understood, and data about its actions on human keratinocytes are scarce.

Objectives and hypothesis

Based on the above findings, we investigated the possible inhibitory actions of the two flavones, luteolin (Fig. 1.1 D) and its structural analogue methoxyluteolin (Fig. 1.1 E), on inflammatory mediator release and cellular proliferation of human mast cells and keratinocytes. We will compare the effects of these two flavones on mast cell activation to the flavonol quercetin (Fig. 1.1 C) and the "mast cell stabilizer" cromolyn (Fig. 1.1 A). In order to determine the mechanism of action of the flavonoids, we will examine several key signaling processes, including intracellular calcium levels, mitochondrial translocation, intracellular ATP production, as well as activation of the nuclear transcriptional factor NF-κB.

We hypothesized that luteolin and methoxyluteolin are more effective than quercetin, and will potently inhibit activation of both human mast cells and keratinocytes. Results obtained from this work may unravel the mechanisms of action of different flavonoids and provide an alternative therapeutic approach for treating Ps and other inflammatory diseases.

Chapter 2. Flavones Block Human Mast Cell Mediator Release

Introduction

Mast cells are immune cells derived from the bone marrow and mature in tissues under the influence of tissue microenvironments[1, 2]. Mast cells are commonly found in tissues interfacing with the external environment, including the skin, respiratory tract and intestines[7]. In addition to the allergic trigger IgE, mast cells can be triggered by bacterial or viral antigens, cytokines, hormones and neuropeptides, such as substance P (SP)[7, 18]. Upon stimulation, mast cells release preformed mediators (histamine, LTs, PGD_2, proteolytic enzymes and preformed TNF) through rapid degranulation (5-30 min)[21-24], as well as newly-synthesized cytokines and chemokines[17], including IL-6, IL-13, IL-8 (or CXCL8), chemokine (C-C motif) ligand 2 (CCL2), as well as vascular endothelial growth factor (VEGF) [11, 191](6-24 hours later). These mediators contribute to the late-phase reactions and to inflammation through the recruitment and activation of other immune cells[17, 192].

Increasing evidence indicates the critical involvement of mast cells in a number of inflammatory diseases, including asthma, autism, multiple sclerosis, interstitial systitis, obesity and Ps[7, 45]. Mast cell-derived histamine and PGD_2 induces bronchoconstriction and mucus secretion, contributing to asthma[46]. Mast cell-derived histamine also induces matrix metalloproteinase-9 expression in keratinocytes and promotes collagen type IV degradation in the basement membrane[47]**.** Mast cells uniquely store preformed TNF[15], which is rapidly released upon stimulation and influences T cell recruitment and

activation[48]. Mast cell-derived CCL2 and IL-8 enhance recruitment of immune cells to the site of inflammation[49]. Mast cell-derived VEGF increases local vascular permeability and promotes angiogenesis[50]. In addition, stimulated live mast cells secrete mitochondrial components, which may act as "autopathogens" contributing to the pathogenesis of inflammatory diseases[45, 51]. With the ability to rapidly release multiple mediators, mast cells actively interact with other cell types in their surrounding environment, participating in the induction and/or propagation of various immune and inflammatory responses. Therefore, inhibition of mast cell activation has clear therapeutic potential.

Currently there are no *effective* mast cell inhibitors. The only clinically available "mast cell stabilizer" cromolyn (Fig. 1.1 A) inhibits rat peritoneal mast cell histamine secretion[137], but could not inhibit rat mucosal mast cells[138, 139], and is a very weak inhibitor of human mast cells[140, 141]. Several studies even reported development of contact dermatitis from cromolyn-containing eyedrops[145, 146]. As a result, there is urgent need for developing effective inhibitors of human mast cells.

In search for potential mast cells inhibitors, we found out that the structure of cromolyn is very similar to the backbone of flavones (Fig. 1.1 B). Flavones belong to a group of naturally occurring polyphenolic compounds called flavonoids, which have potent anti-inflammatory and mast cell blocking activities[147-150]. The flavonol quercetin (Fig. 1.1 C) inhibits rat peritoneal mast cell histamine release by 75% at 10 μM, while 1000 μM of cromolyn is required for comparable inhibition[193]. Quercetin also inhibits histamine, IL-6, IL-8, TNF and tryptase release from human mast cells[188, 194], as well as

asthma development in an animal model[195]. Quercetin and its structurally related flavone luteolin (Fig. 1.1 D) inhibit the release of histamine, LTs and PGD_2 from human cultured mast cells in response to cross-linkage of high affinity FcεRI receptor[154]. Luteolin also inhibits mercury-induced VEGF release from human mast cells[190]. However, the inhibitory effect of different flavonoids has never been compared to that of cromolyn on human mast cell mediator release. In addition, the precise action of flavonoids on human mast cells is not fully understood.

Here we investigated the mast cell blocking effects of various flavonoids. We chose the flavonol quercetin (Fig. 1.1 C) and the flavone luteolin (Fig. 1.1 D) because previous work had shown they are the most potent mast cell inhibitors with the only structural difference being the presence of a hydroxyl group on the center ring. We also used methoxyluteolin (Fig. 1.1 E), which is a structural analogue of luteolin and is more lipid-soluble. We also included the "water-soluble quercetin" (WSQ, Fig. 1.1 F), a quercetin chalcone in which at least the 5,7-hydroxyl groups are in their sodium salt. Cromolyn (Fig. 1.1 A) is included as well because it is known as the mast cell "stabilizer". We first compared the ability of quercetin and cromolyn to inhibit key mediator release from human cultured mast cells. We next investigated whether the quercetin-related flavone luteolin, and its structural analogue methoxyluteolin, inhibits inflammatory mediator production in human mast cells. In order to determine the mechanism of action of the flavonoids, we further examined several key signaling processes involved in mast cell activation and mediator production, including intracellular calcium levels, mitochondrial translocation, intracellular ATP production, as

well as activation of the nuclear transcriptional factor NF-κB, which is known to be involved in mediator synthesis.

Methods

Drugs and Reagents

Recombinant human stem cell factor (rhSCF) was kindly donated by Biobitrum AB, (Stockholm, Sweden). Human IgE was obtained from Millipore (Billerica, MA). Anti-IgE was purchased from Life technologies (Carlsbad, CA). IL-33 was purchased from R&D Systems (Minneapolis, MN) and dissolved in double-distilled water with 0.1% BSA. Cromolyn, quercetin, luteolin, and SP were obtained from Sigma-Aldrich (St Louis, MO). Water-soluble quercetin (WSQ) at pH 7.4 or higher was provided by Thorne Research, Inc. (Dover, ID). Methoxyluteolin was obtained from Pharmascience Nutrients (Clear Water, FL). All flavonoids were dissolved in dimethyl sulfoxide (DMSO), except for WSQ, which was dissolved in double-distilled water forming a solution of pH≈9. Working dilutions for all compounds were prepared in culture media immediately before use. Final concentration of DMSO was < 1% and pH was 7.4. Rabbit anti-NF-κB antibody was purchased from Millipore (Billerica, MA), rabbit anti-actin antibody and the secondary HRP-conjugated antibody were purchased from Cell Signaling Technology (Beverly, MA).

Human mast cell culture

The limited number of mast cells obtained from normal human tissues has led to increasing use of human leukemic cultured LAD2 mast cells [196] or primary hCBMCs previously reported to release cytokines, histamine and tryptase in response to anti-IgE[197, 198]. Human LAD2 mast cells derived from a human mast cell leukemia [196] (kindly

supplied by Dr. A Kirshenbaum, NIH, Bethesda, MD) were cultured in StemPro-34 medium (Life Technologies) supplemented with 2 mM L-gLuteolinamine, 1% penicillin/streptomycin and 100 ng/mL rhSCF (Biobitrum AB).

In order to obtain primary hCBMCs, human umbilical cord blood was obtained during normal deliveries in accordance with established institutional guidelines. Mononuclear cells were isolated by layering heparin-treated cord blood onto Lymphocyte Separation Medium (INC Biomedical, Aurora, OH). CD34+ progenitor cells were isolated from mononuclear cells by positive selection of AC133 (CD133+/CD34+) cells by magnetic cell sorting (CD133 Microbead Kit, Miltenyi Biotech, Auburn, CA). For the first six weeks, CD34+ progenitor cells were cultured in IMDM medium (Life Technologies) supplemented with 0.1% BSA, 1% insulin-transferin-selenium, 50 ng/mL IL-6, 0.1% β-mercaptoethanol, 1% penicillin/streptomycin and 100 ng/mL rhSCF. After six weeks the cells were cultured in IMDM supplemented with 10% FBS, 50 ng/mL IL-6, 0.1% β-mercaptoethanol, 1% penicillin/streptomycin and 100 ng/mL rhSCF. Cells were maintained at 37ºC in a humidified incubator at 95% O_2/5% CO_2 atmosphere. hCBMCs cultured for at least 15 weeks were used for experiments and cell purity was above 98%. Cell viability was determined by trypan blue (0.4%) exclusion.

Degranulation assays

The release of β-hex, as an index of mast cell degranulation, was assayed using a fluorometric assay. LAD2 mast cells (0.5×10^5) were preincubated with various drugs (1-100 μM) for 30 min and subsequently triggered with SP (2 μM) for 30 min. Control cells

were treated with 0.1% DMSO. Supernatant fluids were collected and cell pellets were lysed with 1% Triton X-100. Supernatant fluids and cell lysates were incubated in the reaction buffer (p-nitrophenyl-N-acetyl-β-D-glucosaminide from Sigma) for 1.5 hour (h) and then 0.2 M glycine was added to stop the reaction. Absorbance was measured at 405 nm in an ELISA plate reader. The results are expressed as the percentage of β-hex released over the total.

Mast cell degranulation was also assessed by measuring histamine, PGD_2 and LTs release in the supernatant fluid 30 min after cell stimulation. Histamine levels were assayed using a SPI Bio histamine EIA kit (Bertin Pharma, France). PGD_2 release was measured using a PGD_2-MOX EIA kit (Cayman Chemical Co., Ann Arbor, MI). LTs levels were assayed using a Luminex cysteinyl leukotriene kit (Cayman Chemical Co.) according to the manufacturer's instructions.

To assay degranulation in primary human mast cells, hCBMCs (0.5×10^5) were first primed with human IgE (1 μg/mL, Millipore) overnight and preincubated with various drugs (1-100 μM) for 30 min before stimulation with anti-IgE (10 μg/mL, Life technologies) for 30 min. Supernatant fluids were collected and preformed mediator release was measured as described above.

Mediator release

For CCL2 and IL-6 release, hCBMCs (1×10^5 cells) were primed with human IgE (1 μg/mL, Millipore) overnight and preincubated with various flavonoids (1-100 μM) for 30 min before stimulation with anti-IgE (10 μg/mL, Life technologies) for 24 h. CCL2 and IL-6 were measured in the supernatant fluids using Enzyme-Linked Immunosorbent Assay (ELISA) kits according to the instructions provided (R&D Systems).

For TNF and IL-8 release, LAD2 cells (1×10^5 cells) were preincubated with various flavonoids (1-100 μM) for different times as indicated, and subsequently triggered with SP (2 μM) for 24 h. Release of TNF and IL-8 was measured in the supernatant fluids using commercially available ELISA assay kits (R&D Systems).

RNA isolation and quantitative real time PCR (qRT-PCR)

LAD2 cells and hCBMCs (5×10^5 cells) were treated the same way as described above with various drugs before stimulation with either SP (2 μM) or anti-IgE (10 μg/mL) for 6 h. Total RNA was extracted with an RNeasy Mini kit (Qiagen Inc., Valencia, CA) in accordance with the manufacturer's specifications. An iScript cDNA synthesis kit (BioRad, Hercules, CA) was used for reverse-transcription of each sample. Quantitative RT-PCR was performed using Taqman gene expression assays (Applied Biosystems, Foster City, CA) for TNF (Hs99999043_m1) and CCL2 (Hs00234140_m1), as well as the two genes encoding two different subunits of the NF-κB protein complex, NFKB1 (NF-κB p50 subunit, Hs00765730_m1) and RELA (NF-κB p65 subunit, Hs00153294_m1). Samples were run at 45 cycles using a real-time PCR system (7300,

Applied Biosystems). Relative mRNA expression was determined from standard curves run with each experiment. The mRNA gene expressions were normalized to GAPDH endogenous control (4310884E, Applied Biosystems).

Intracellular calcium measurements

Intracellular calcium ion levels were measured at 37 °C using Fura-2AM as indicator. LAD2 cells were suspended in Tyrode's buffer with 30 nM Fura-2 AM (Life Technologies) for 20 min to allow Fura-2AM to enter the cells. After centrifugation and washing to remove excess dye, the cells were resuspended in plain Tyrode's buffer and incubated for another 20 min. Cells were then transferred to 96-well plates (1×10^5 cells per well). In some experiments, cells were pretreated with various flavonoids (10-100 μM) for 30 min. Cells were then triggered with SP (2 μM). Chanegs in Fura-2 fluorescence was immediately read by MDC FlexStation II (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 340 nm/380 nm and emission wavelength of 510 nm. Results were processed according to the Life Technologies Fura-2AM protocol and reported as relative ratio.

Confocal microscopy

The "lysosome" dye LysoTracker [199] was used to stain mast cell secretory granules because their average pH is about 5.5[200], which is similar to that of the lysosomes. Human mast cells were incubated with 20 nM MitoTracker deep red probe (Invitrogen) for 20 min and 50 nM LysoTracker DND (Invitrogen) for 30 min. Cells were washed, moved to glass bottom culture dishes (MatTek, Ahsland, MA, USA) and

observed using a Leica TCS SP2 Confocal microscopy (Leica, Buffalo Grove, IL, USA). The percentage of cells with mitochondrial translocation was counted from 100 randomly selected mast cells in each experiment by three independent operators. Confocal digital images were processed using the National Institute of Health ImageJ 1.32 and Adobe Photoshop 7.0 Programs.

Intracellular ATP measurement

In order to determine if luteolin and methoxyluteolin have any effect on cellular energy production, intracellular ATP content was measured. After incubation with luteolin or methoxyluteolin (50, 100 μM) for up to 24 h, 1×10^6 LAD2 cells were lysed and intracellular ATP contents were determined using an ATP assay kit (Abcam, Cambridge, MA) according to the instructions provided.

NF-κB nuclear translocation by Western blot analysis

After preincubation with different drugs (100 μM, 30 min), LAD2 mast cells (2×10^5 cells per well) were stimulated with SP (2 μM) for 60 min. Nuclear fractions were isolated using a NE-PER nuclear extraction kit (Thermo Scientific, Rockford, IL). Changes in total NF-κB protein levels in both cytosolic and nuclear fractions were detected by Western blot analysis. The protein concentrations were determined using Bio-Rad Protein Assay reagent, and equal amounts of protein were subjected to Western blotting by using the indicated antibodies. Briefly, samples separated by SDS/PAGE were transferred to nitrocellulose membranes. After being blocked in 5% BSA (w/v) at room temperature for 1 h, the membranes were rinsed and incubated at 4 °C overnight

with a variety of primary antibodies (1:1,000 dilution). The membranes were then washed and incubated with secondary antibody (1:2,000 dilution) at room temperature for 1 h, developed with chemiluminescence ECL reagent (LumiGold, SignaGen Laboratories, Gaithersburg, MD), and exposed to Hyperfilm MP (GE Healthcare, Piscataway, NJ).

NF-κB p65 DNA-binding activity by ELISA

After luteolin and methoxyluteolin preincubation (10-100 μM, 6 h), LAD2 cells (2×10^6 cells) were stimulated with SP (2 μM, 15 min). Cells were then harvested and cytosolic and nuclear extracts were isolated as described above. DNA-binding activity of NF-κB p65 was detected by the NF-κB (p65) Transcription Factor Assay Kit (#10007889, Cayman Chemical Co.) according to the instructions provided. Cytosolic and nuclear extracts (10 μg of protein) were added to a 96-well plate coated with a specific double stranded DNA sequence containing the NF-κB response element. NF-κB was detected by addition of specific primary antibody directed against NF-κB followed by HRP-conjugated secondary antibody to provide a colorimetric readout at 450 nm. Relative NF-κB p65 DNA-binding activities were normalized to control cells treated with 0.1% DMSO only.

Statistical analysis

All experimetns were performed in triplicate and were repeated for at least three times (n=3). Results are presented as mean ± SD. Data between different treatment

groups were compared using the unpaired, 2-tailed, Student's *t*-test. Significance of comparisons is denoted by * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Results

Quercetin blocks mediator release from primary hCBMCs

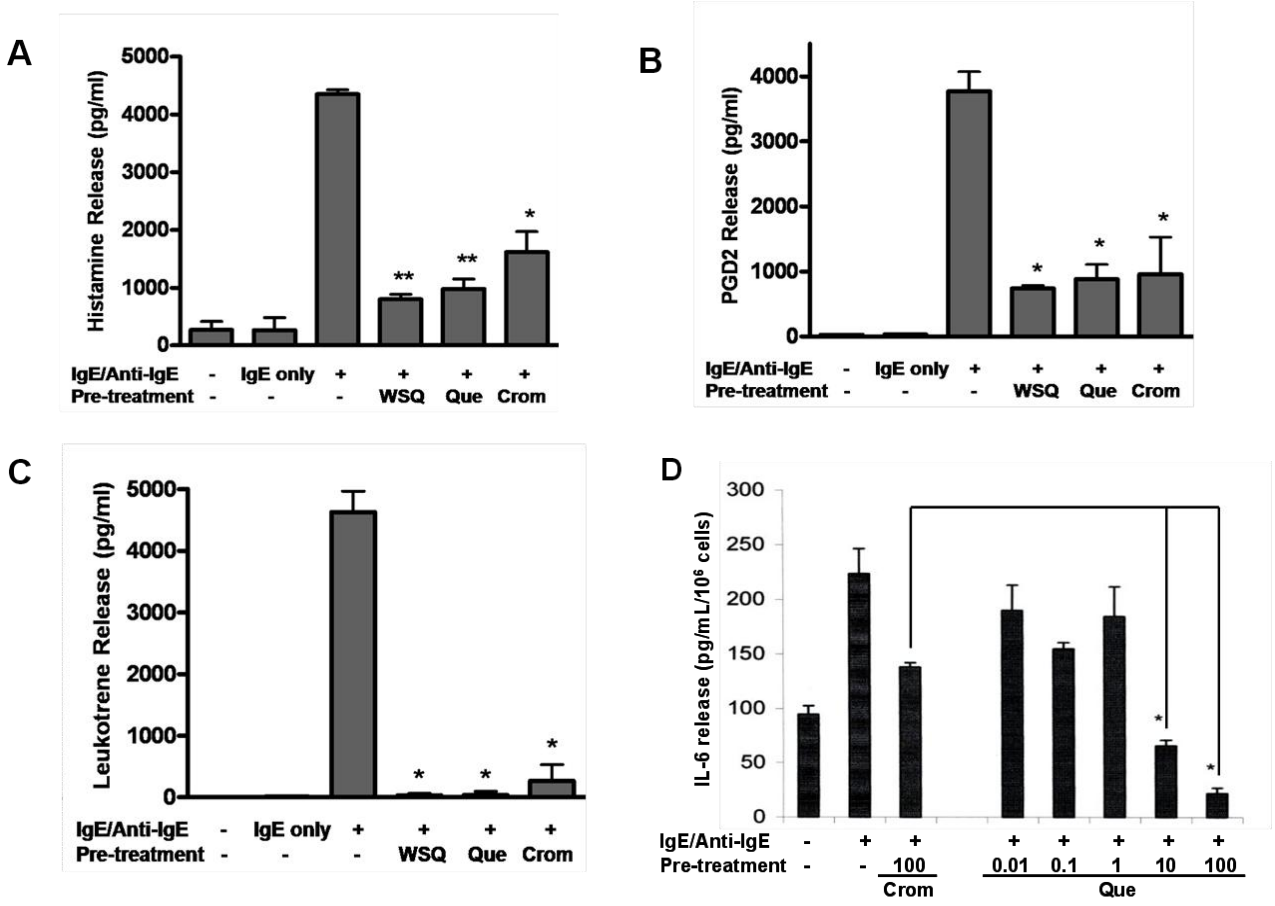
Human mast cells can be activated by cross-linking of IgE bound to its high affinity receptors, which is achieved by overnight priming of mast cells with IgE before subsequent stimulation with anti-IgE. We measured the release of preformed mediators (histamine, PDG_2 and LTs) from hCBMCs as an index of degranulation. Stimulation with IgE/anti-IgE triggers rapid release of large amounts of histamine, PDG_2 and LT from hCBMCs compared to control cells primed with IgE only (Fig. 2.1, A-C). Preincubation with quercetin or WSQ significantly reduces histamine secretion (Fig. 2.1 A) from 4347.9 to 977.3 pg/mL (82% inhibition), and to 797.7 pg/mL (87% inhibition), respectively, compared to cromolyn (1613.6 pg/mL, 67% inhibition). Quercetin and WSQ inhibit PGD_2 release (Fig. 2.1, B) from 3771.8 to 882.5 pg/mL (77% inhibition), and to 740.8 pg/mL (81% inhibition), respectively, compared to cromolyn (958.3 pg/mL, 75% inhibition). In addition, quercetin and WSQ block LT secretion (Fig. 2.1, C) from 4628.6 to 35.3 pg/mL (99% inhibition), and to 29.7 pg/mL (99% inhibition), respectively, compared to to cromolyn (530.9 pg/mL, 88% inhibition).

Apart from preformed mediators, primary hCBMCs also secrete newly-synthesized IL-6 (223.5 pg/mL) 24 h after stimulation with IgE/anti-IgE, and is inhibited by quercetin in a concentration-dependent manner (Fig. 2.1, D). Quercetin preincubation at 10 and 100 μM for 30 min reduces IL-6 release to 65.8 pg/mL and 29.7 pg/mL, respectively, which is even lower than that of control (96.8 pg/mL). Cromolyn (100 μM, 30 min) is able to decrease IL-6 release to 141.3 pg/mL.

It should be noted that 30 min preincubation with cromolyn has no effect due to rapid tachyphylaxis. Instead, cromolyn has to be added *together* with the trigger for any inhibitory effect to be evident. On the other hand, quercetin and WSQ are also effective when added 30 min prior to the trigger.

Figure 2.1. Effects of quercetin and cromolyn on mediator release from primary hCBMCs

hCBMCs were incubated with IgE (1 μg/mL) overnight and then stimulated with anti-IgE (10 μg/mL) for 2 h to measure release of (A) Histamine, (B) PGD_2 and (C) LTs. In some experiments, hCBMCs were preincubated with various drugs (100 μM) for 30 min before stimulation with anti-IgE. In (D), hCBMCs were preincubated with either 100 μM cromolyn or increasing concentrations of quercetin for 30 min before anti-IgE stimulation. IL-6 release was measured in the supernatant fluids by ELISA. WSQ, water soluble quercetin; Que, quercetin; Crom, cromolyn. Statistical significance between pre-treatment and stimulation groups was denoted as * $p<0.05$, ** $p<0.01$.

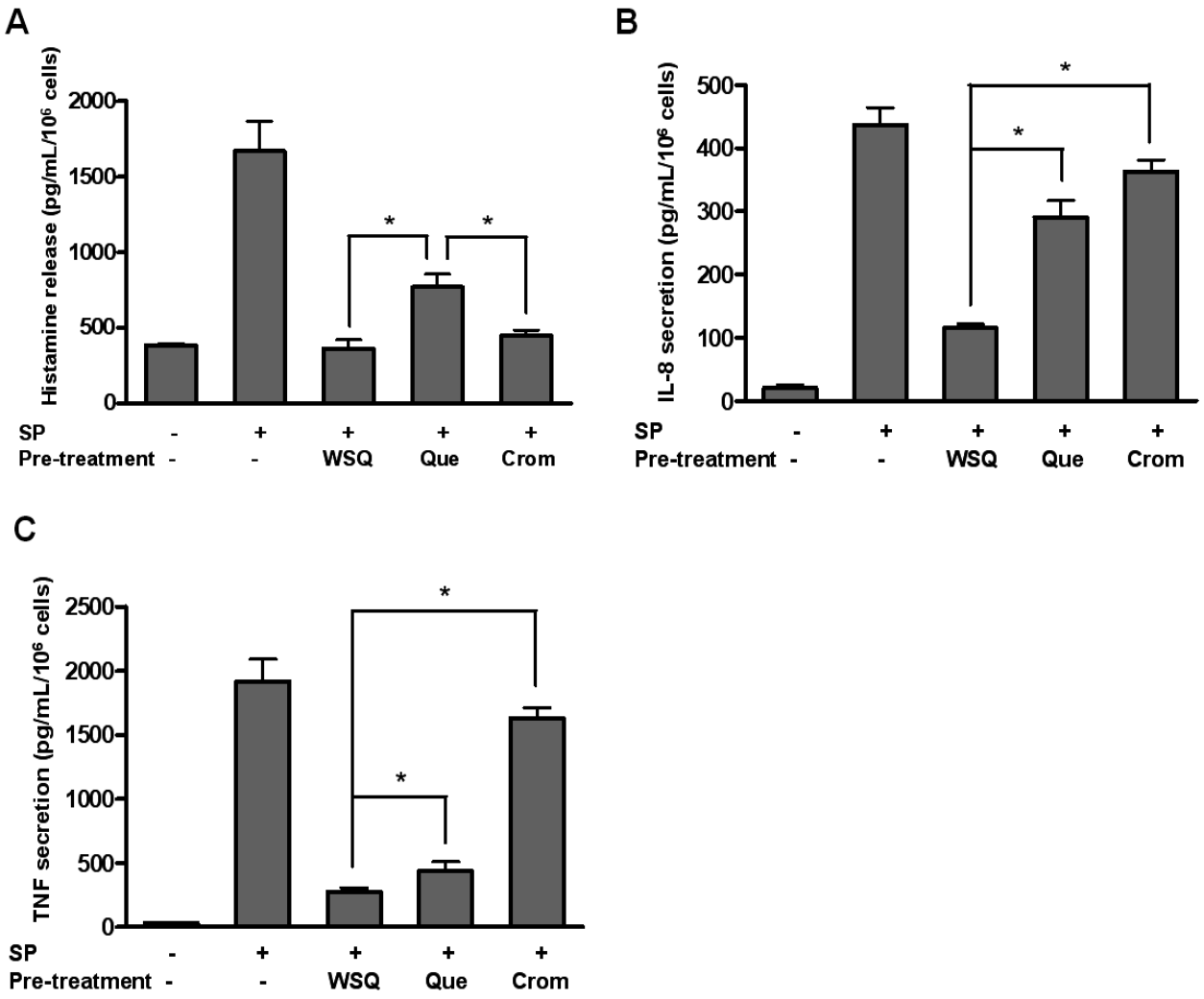


Quercetin is more effective than cromolyn in blocking mediator release from human leukemic LAD2 mast cells

Apart from the allergic trigger IgE/anti-IgE, human mast cells can also be activated by the neuropeptide substance P (SP). LAD2 cells release histamine 2 h after SP (2 μM) stimulation (1611.4 pg/mL, Fig. 2.2, A). WSQ inhibits histamine release to 350.6 pg/mL, which is even lower than that of control (388.7 pg/mL). Quercetin and cromolyn reduce histamine release to 792.3 pg/mL and 464.6 pg/mL, respectively (Fig. 2.2, A). LAD2 cells also secrete newly-synthesized IL-8 and TNF 24 h after SP stimulation. Preincubation with WSQ, quercetin and cromolyn (100 μM, 30 min) block IL-8 secretion from 437.2 pg/mL to 115.4 pg/mL, 291.2 pg/mL and 362.9 pg/mL, respectively. Similarly, WSQ, quercetin and cromolyn (100 μM, 30 min) block TNF secretion from 1917.2 pg/mL to 274.7 pg/mL, 436.9 pg/mL and 1628.8 pg/mL, respectively (Fig. 2.2, B, C). WSQ is the most effective while cromolyn is the least effective.

Figure 2.2. Effects of quercetin, WSQ and cromolyn on mediator release from human leukemic LAD2 mast cells

LAD mast cells were pre-treated with various flavonoids (100 µM, 30 min) before stimulation with SP (2 µM). Cromolyn was added at the same time as SP stimulation. (A) Histamine release was measured 30 min after SP stimulation; (B) IL-8 and (C) TNF were measured in the supernatant fluids 24 h after SP stimulation by ELISA. * $p<0.05$.



Luteolin is more effective than quercetin in blocking degranulation of human LAD2 mast cells

In addition to quercetin, we studied the effects of luteolin, and its structural analog methoxyluteolin, on human mast cell activation. We first compared the inhibitory effects of cromolyn, quercetin and luteolin on SP-triggered degranulation of LAD2 cells.

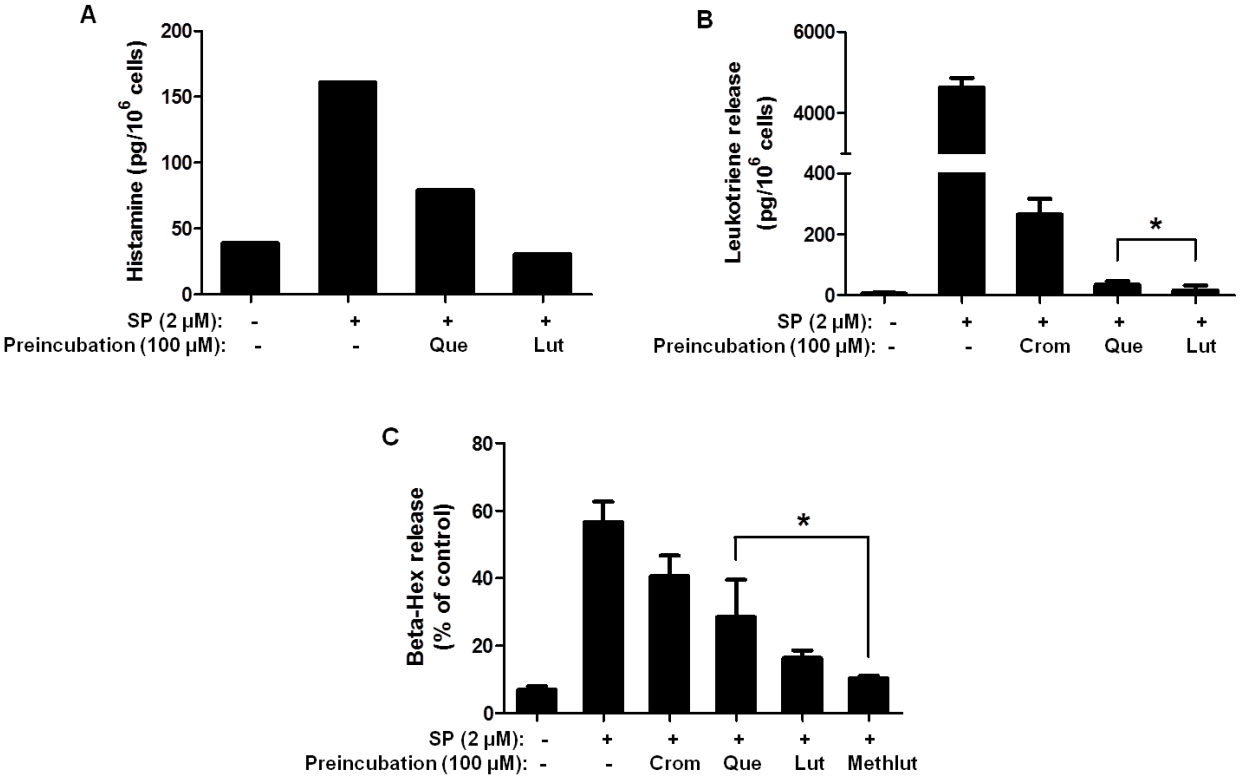
We measured the release of preformed mediators (histamine, LT and β-hex) from LAD2 cells as an index of degranulation. Stimulation with SP triggers rapid release of histamine (Fig. 2.3 A). Preincubation with luteolin (100 μM, 30 min) completely blocks histamine release, while the same preincubation with quercetin (100 μM, 30 min) only reduces histamine release from 161 pg/mL to 79 pg/mL (50 % inhibition) (Fig. 2.3 A, n=1).

SP triggers LTs release from LAD2 cells, which is reduced by cromolyn by 88% (Fig. 2.3 B). Quercetin and luteolin block LTs release from 4628 pg/m to 35 pg/mL (99% inhibition), and to 16 pg/mL (99% inhibition), respectively. Luteolin blocks significantly more LTs release than quercetin (Fig. 2.3 B).

SP also triggers β-hex release from LAD2 cells, which is decreased by cromolyn and quercetin by 20 % and 44 %, respectively (Fig. 2.3 C). Luteolin decreass β-hex release by 80 %. Methoxyluteolin is the strongest inhibitor and almost completely blocks β-hex release from LAD2 cells (Fig. 2.3 C).

Figure 2.3. Effects of quercetin, luteolin and methoxyluteolin on degranulation of human LAD2 mast cells

LAD2 cells were preincubated with various drugs (100 μM, 30 min) before stimulation with SP (2 μM, 30 min). Release of (A) Histamine (n=1); (B) LTs and (C) β-hex (n=3). * $p<0.05$. Crom, cromolyn; Que, quercetin; Lut, luteolin; Methlut, methoxyluteolin.



Methoxyluteolin is more effective than luteolin in blocking human mast cell degranulation

Luteolin and methoxyluteolin have the strongest inhibitory actions on mast cell degranulation. We further studied the effects of these two flavones on human mast cell activation and mediator release.

The release of β-hex, preformed TNF and histamine in LAD2 cells within 30 min of trigger stimulation was assayed. SP (2 μM) triggers 40% β-hex release from LAD2 cells. Preincubation with luteolin or methoxyluteolin (10-100 μM) concentration-dependently inhibits SP-triggered β-hex release, with almost complete inhibition at 100 μM (Fig. 2.4 A).

SP stimulates significant release of preformed TNF from LAD2 cells (Fig. 2.4 B), which is also inhibited in a concentration-dependent manner by luteolin and methoxyluteolin. Preincubation with methoxyluteolin (10 μM, 30 min) significantly decreases preformed TNF release from 285 pg/10^6 cells to 186 pg/10^6 cells, which is more effective than luteolin. Similarly at 100 μM, methoxyluteolin inhibits preformed TNF release by 95%, whilst luteolin reaches only 60% inhibition.

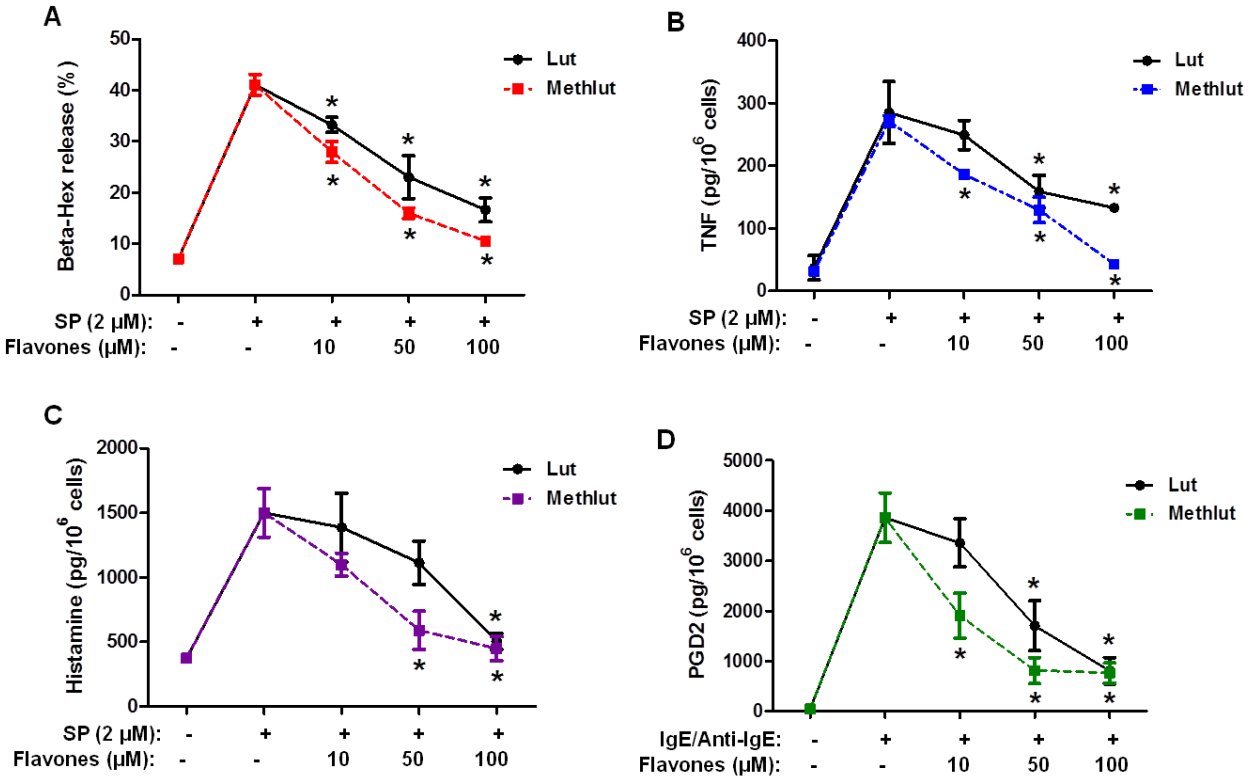
LAD2 cells also release histamine in response to SP (Fig 2.4 C). Methoxyluteolin reduces histamine release from 1501 pg/10^6 cells to 590 pg/10^6 cells at 50 μM, which is more effective than luteolin (1114 pg/10^6 cells). Both flavones significantly inhibit histamine release by more than 90% at 100 μM.

In primary hCBMCs, IgE/anti-IgE stimulates large amount of PGD_2 (3855 pg/10^6 cells) within 30 min compared to control cells treated with IgE only (44 pg/10^6 cells, Fig. 2.4 D). At 10 μM, luteolin is not effective in inhibiting PGD_2 release, while methoxyluteolin significantly decreases PGD_2 release by 52%. Both luteolin and methoxyluteolin block PGD_2 release by more than 80% at 100 μM.

In summary, luteolin and methoxyluteolin strongly inhibit human mast cell degranulation. Methoxyluteolin is more effective than luteolin.

Figure 2.4. Effects of luteolin and methoxyluteolin on human mast cell degranulation

LAD2 cells were triggered with SP (2 μM, 30 min). Primary hCBMCs were incubated with IgE (1 μg/mL) overnight and then triggered with anti-IgE (10 μg/mL, 30 min). In some experiments, cells were preincubated with luteolin or methoxyluteolin (10-100 μM, 30 min) before stimulation. Release of (A) β-hex, (B) preformed TNF, (C) histamine and (D) PGD_2 were assayed in the supernatant fluids. Statistical significance between flavone preincubation and trigger stimulation (SP or IgE/anti-IgE) was denoted as *p<0.05.



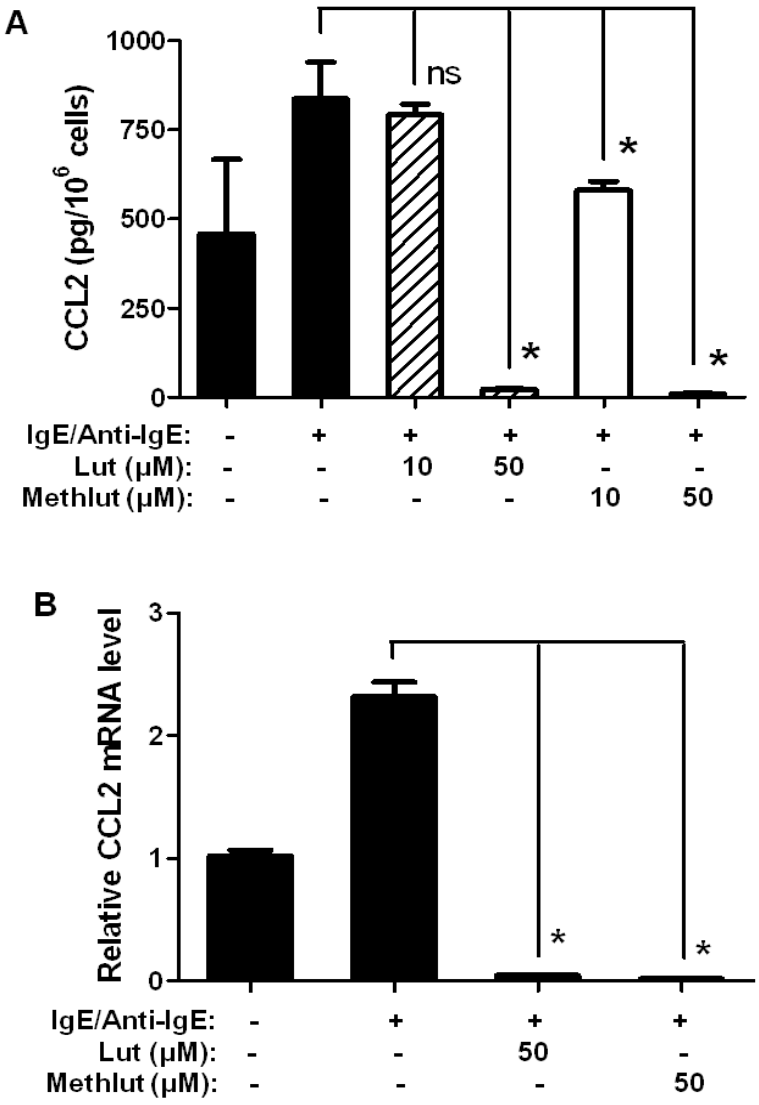
Luteolin and methoxyluteolin inhibit CCL2 production in hCBMCs

Apart from rapid degranulation to release preformed mediators, we also studied the effects of luteolin and methoxyluteolin on delayed release of newly-synthesized cytokines and chemokines from human mast cells. IgE/Anti-IgE (10 μg/mL, 24 h) triggers CCL2 production from primary hCBMCs. Luteolin or methoxyluteolin preincubation (50 μM, 30 min) completely blocks CCL2 release (Fig. 2.5 A).

Stimulation with IgE/anti-IgE (10 μg/mL, 6 h) also triggers 2-fold increase in CCL2 mRNA expression compared to control cells primed with IgE only (Fig. 2.5 B). Preincubation with luteolin or methoxyluteolin (50 μM, 30 min) completely blocks IgE/anti-IgE-triggered mRNA gene expression of CCL2. We chose to examine mRNA expression at 6 h after trigger stimulation because this is the adequate time point to detect significant differences in mRNA expression level.

Figure 2.5. Effects of luteolin and methoxyluteolin on IgE/anti-IgE-triggered CCL2 production in hCBMCs

After overnight priming with IgE (1 μg/mL), hCBMCs were incubated with luteolin or methoxyluteolin (50 μM, 30 min) before stimulation with anti-IgE (10 μg/mL, 24 h). (A). CCL2 release was assayed by ELISA in the supernatant fluids. (B) hCBMCs were incubated with luteolin or methoxyluteolin (50 μM, 30 min) before stimulation with anti-IgE (10 μg/mL, 6 h). Total RNA was isolated and mRNA expression of CCL2 was examined by quantitative RT-PCR. *p<0.05.



Luteolin decreases IL-33-triggered TNF production in LAD2 mast cells

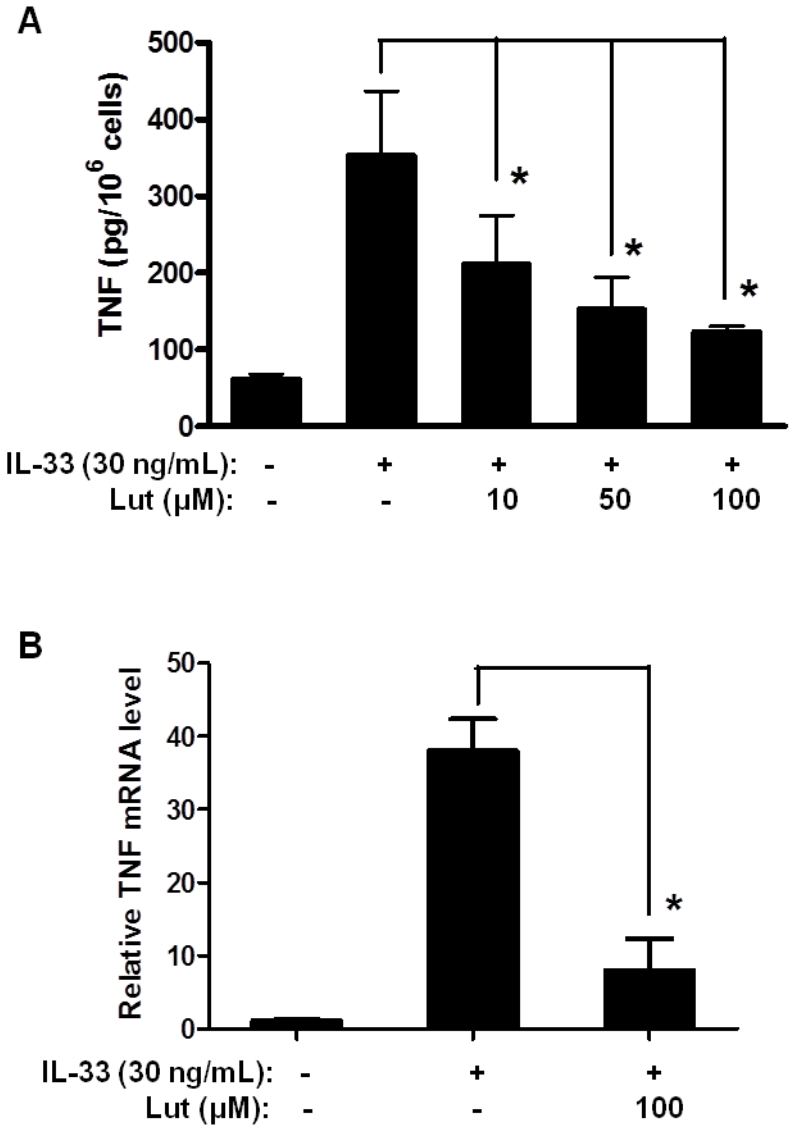
The cytokine IL-33 has been shown to stimulate pro-inflammatory mediator release from human mast cells[88]. Moreover, IL-33 is secreted by psoriatic keratinocytes and induces cytokine release from HMC-1 human mast cells[201]. IL-33 is also increased in Ps lesional skin[93], indicating its potential role in mast cell-keratinocyte interactions and participation in Ps pathogenesis.

In our studies, IL-33 treatment (30 ng/mL, 24 h) triggers significant TNF release (352 pg/10^6 cells) compared to control cells treated with DMSO only (61 pg/10^6 cells, Fig. 2.6 A). This effect is concentration-dependently inhibited by preincubation with luteolin (10-100 μM, 30 min).

IL-33 stimulation (30 ng/mL, 6 h) also triggers 37-fold increase in TNF mRNA expression compared to control cells (Fig. 2.6 B). Preincubation with luteolin for 30 min significantly decreases IL-33-triggered TNF mRNA expression by 81%.

Figure 2.6. Effect of luteolin on IL-33-triggered TNF production in LAD2 mast cells

LAD2 cells were preincubated with luteolin (10-100 μM) for 30 min before stimulation with IL-33 (30 ng/mL) for 24 h. (A) TNF release was measured by ELISA. (B) The mRNA expression of TNF was measured by quantitative RT-PCR. * $p<0.05$.



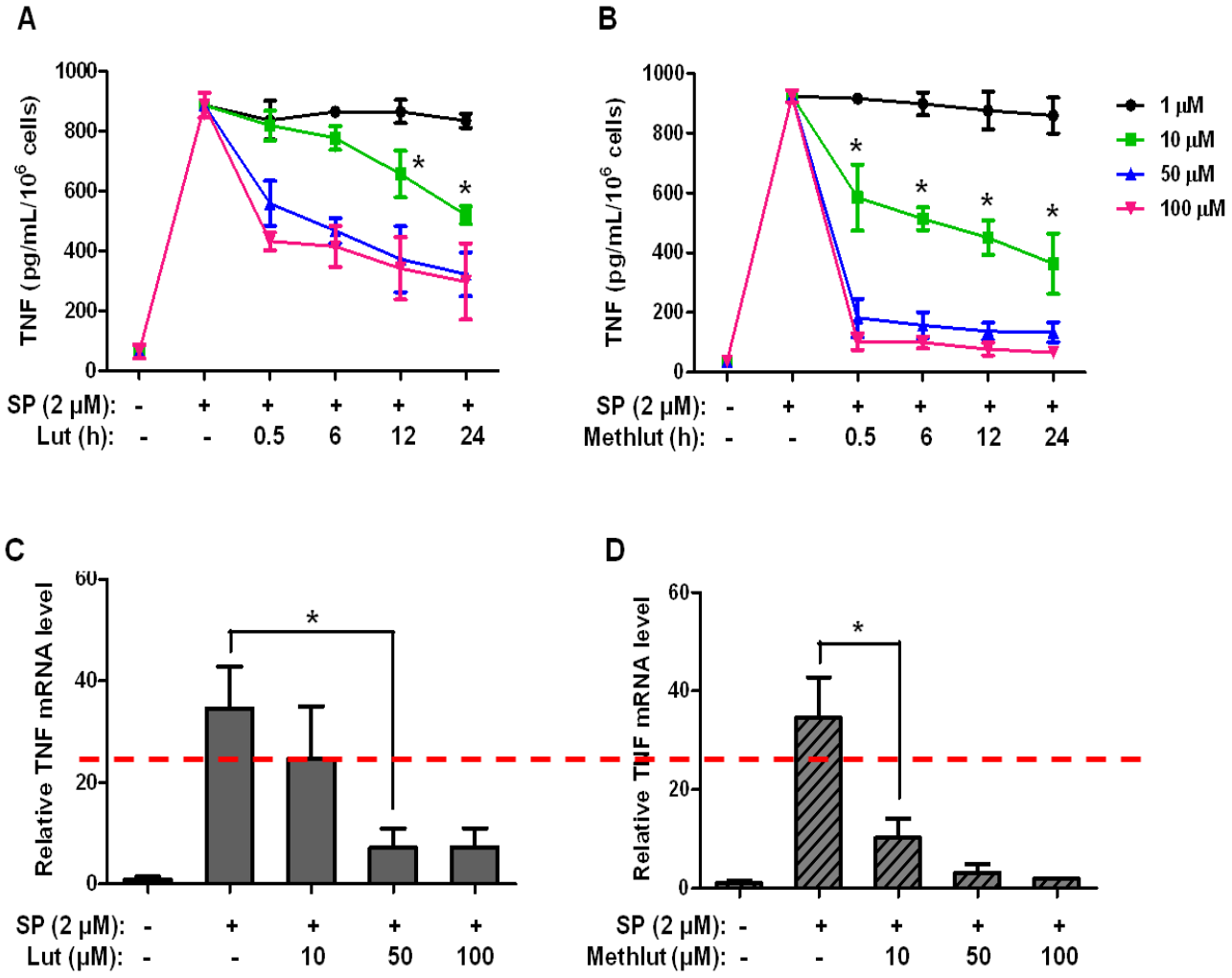
Methoxyluteolin is more effective than luteolin in decreasing SP-triggered TNF production in LAD2 mast cells

In addition to preformed TNF, LAD2 cells also synthesize and release new TNF 6-24 hours later. SP (2 μM, 24 h) triggers significant TNF release (886 pg/10^6 cells) compared to control cells (65 pg/10^6 cells, Fig. 2.7 A), which is inhibited by preincubation with luteolin or methoxyluteolin (10-100 μM, 30 min) in a concentration-dependent manner. Moreover, prolonged preincubation does not increase the extent of inhibition, except for luteolin where inhibition at10 μM becomes more prominent after 12 and 24 h as compared to 30 min (Fig. 2.7 A). Luteolin at 50 and 100 μM reduces TNF release by about 50%, whilst methoxyluteolin achieves 95% inhibition at the same concentrations. Methoxyluteolin is also more effective than luteolin in inhibiting TNF release at 10 μM (Fig. 2.7 B).

Stimulation with SP (2 μM, 6 h) triggers 34-fold increase in TNF mRNA expression compared to control cells (Fig. 2.7 C, D). Preincubation with luteolin or methoxyluteolin for 30 min significantly decreases SP-triggered TNF mRNA expression. At 10 μM, methoxyluteolin is more effective than luteolin and reduces SP-triggered TNF expression by 73% (Fig. 2.7 D), which corresponds to the pattern of TNF protein release measured by ELISA.

Figure 2.7. Effects of luteolin and methoxyluteolin on SP-triggered TNF production in LAD2 mast cells

LAD2 cells were incubated with luteolin (A) or methoxyluteolin (B) (1-100 μM) for up to 24 h before stimulation with SP (2 μM, 24 h). TNF release into the supernatant fluids was assayed by ELISA. In another set of experiments, LAD2 cells were incubated with luteolin (C) or methoxyluteolin (D) (10-100 μM, 30 min) before stimulation with SP (2 μM, 6 h). Total RNA was isolated and mRNA expression of TNF was examined by quantitative RT-PCR. *p<0.05.

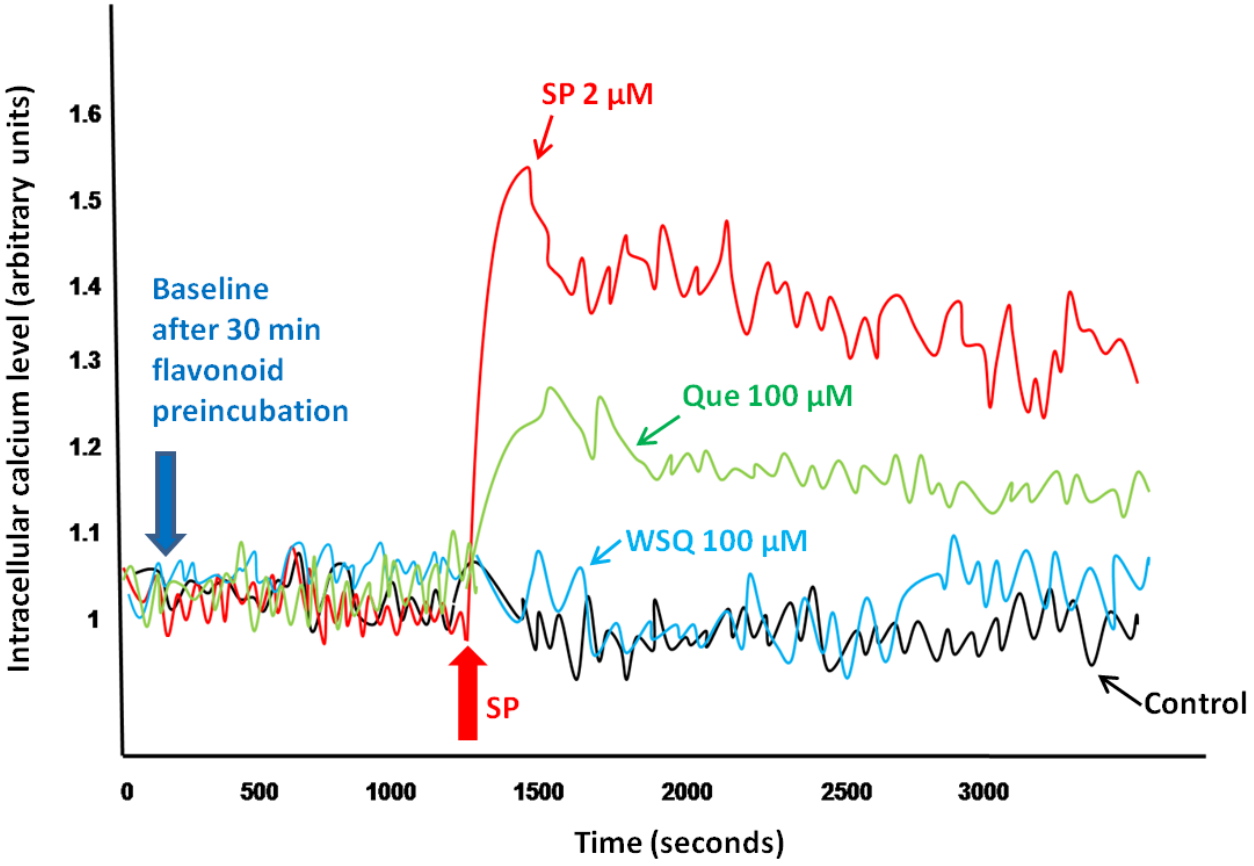


Quercetin inhibits SP-triggered intracellular calcium increase in LAD2 mast cells

As shown above, flavonoids effectively inhibit pro-inflammatory mediator release from human mast cells triggered by different allergic and non-allergic stimulation. In order to investigate their possible mechanism of action, we first studied the effect of quercetin on intracellular calcium ion elevation, which is required for mast cell degranulation. SP triggers rapid increase of intracellular calcium ions in LAD2 cells (Fig. 2.8). Preincubation with WSQ (100 μM, 30 min) completely inhibits the intracellular calcium ion increase as compared to quercetin, which inhibits calcium level by 50% at equimolar concentration (Fig. 2.8). Cromolyn (100 μM) has no effect on calcium levels whether added together with or prior to the trigger (data not shown).

Figure 2.8. Effects of quercetin on SP-stimulated intracellular calcium elevation in LAD2 mast cells

LAD2 cells were loaded with Fura-2 AM (30 nM, 20 min) and then preincubated with quercetin or WSQ (100 µM) for 30 min. Immediately after SP addition (2 µM), Fura-2 fluorescence was monitored for 20 min at an excitation wavelength of 340 nm and 380 nm and emission wavelength of 510 nm. Control cells were treated with DMSO vehicle (0.1%). Que, quercetin; WSQ, water-soluble quercetin.



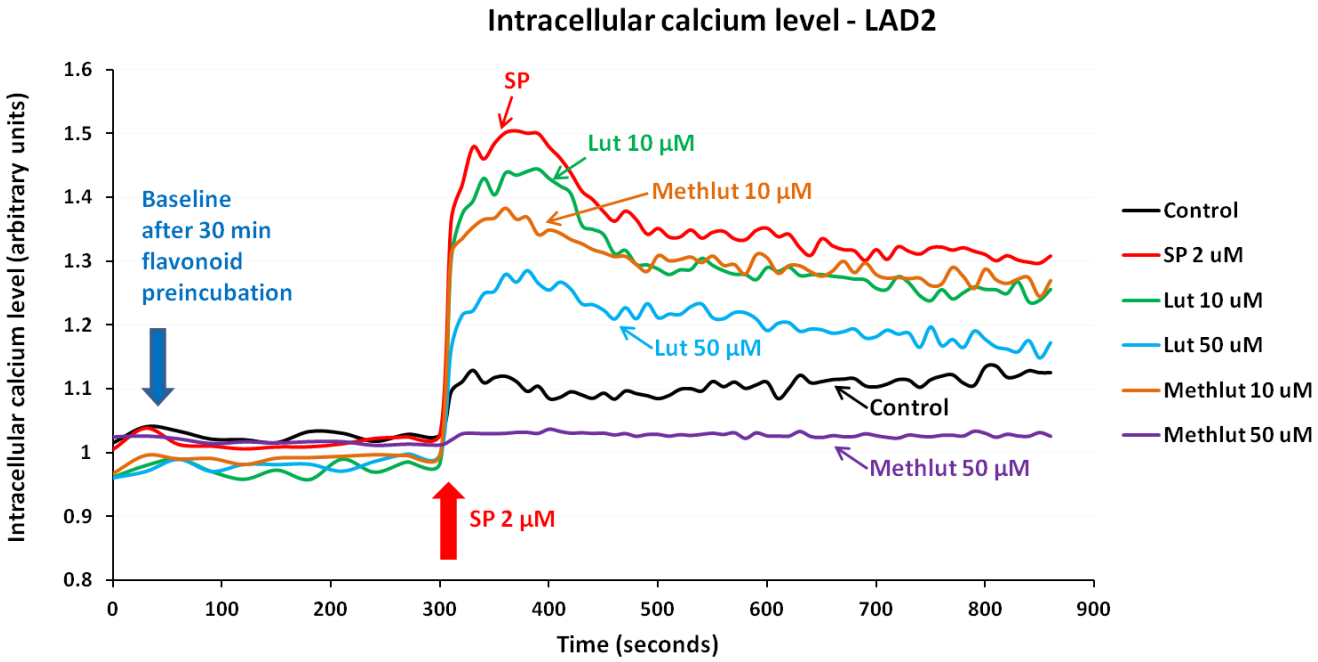
Methoxyluteolin is more effective than luteolin in decreasing intracellular calcium elevation in LAD2 mast cells

We then studied whether luteolin and methoxyluteolin have any effect on intracellular calcium levels. SP (2 μM) again triggers a rapid intracellular calcium increase in LAD2 cells (Fig. 2.9). Preincubation with luteolin at 50 μM for 30 min inhibits calcium increase by about 50%, which is to the the same extent of inhibition achieved by quercetin at a higher concentration (100 μM, Fig. 2.8). Preincubation with methoxyluteolin (50 μM, 30 min) completely blocks intracellular calcium elevation (Fig. 2.9).

Noteworthy, there is a small and sustained calcium increase in control cells treated with DMSO vehicle (0.5%), where methoxyluteolin (50 μM) containing the same amount of DMSO completely blocked calcium elevation, which is even lower than that of DMSO vehicle-treated control cells (Fig. 2.9).

Figure 2.9. Effect of luteolin and methoxyluteolin on intracellular calcium elevation in LAD2 mast cells

LAD2 cells were loaded with Fura-2 AM (30 nM, 20 min) and then preincubated with luteolin or methoxyluteolin (10 and 50 µM) for 30 min. Immediately after stimulation with SP (2 µM), Fura-2 fluorescence was monitored at an excitation wavelength of 340 nm and 380 nm and emission wavelength of 510 nm. Control cells were treated with DMSO (0.1%). Lut, luteolin; Methlut, methoxyluteolin.



Luteolin reduces SP-triggered mitochondrial translocation in LAD2 mast cells

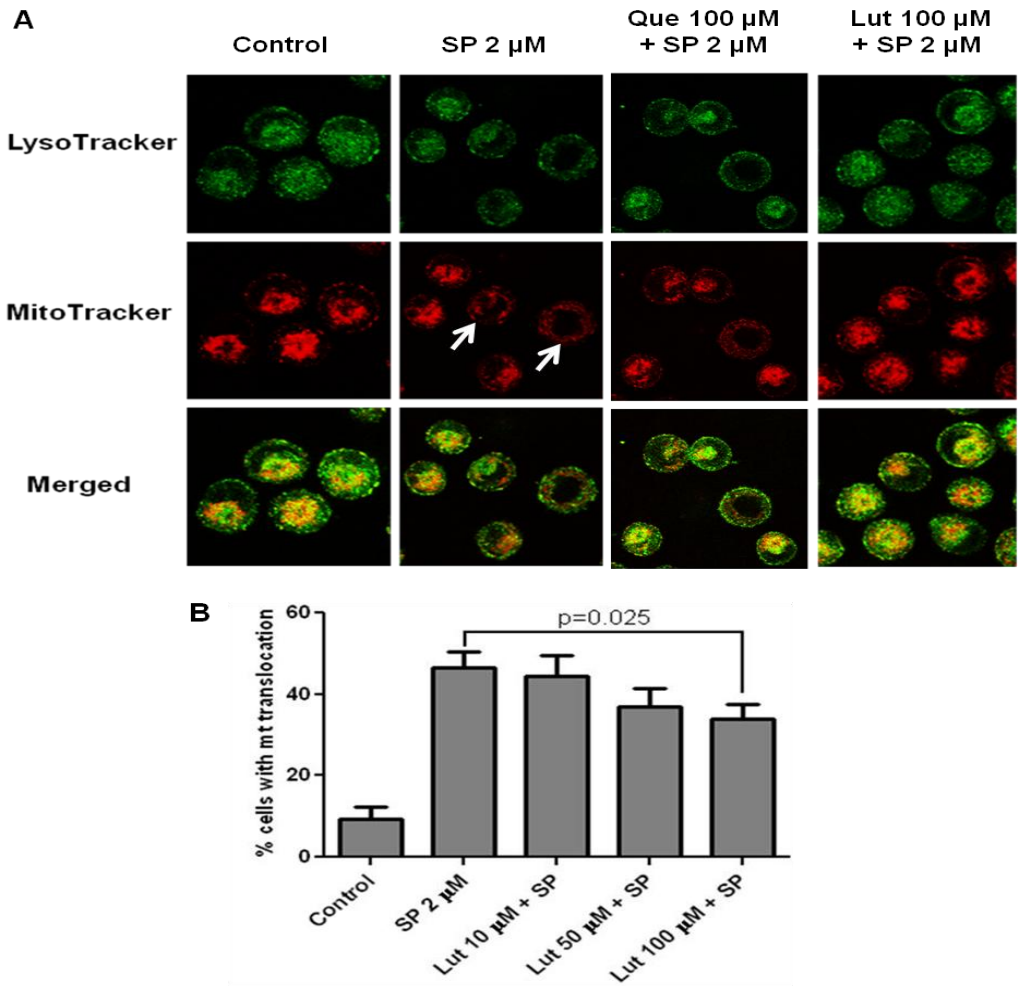
We had previously shown that human mast cell activation is accompanied by mitochondrial fission and translocation from a peri-nuclear region towards the cell membrane. We thus examined effects of luteolin on mitochondrial translocation in SP-triggered LAD2 cells.

LAD2 cells were stained with MitoTracker deep red probe to visualize mitochondria and LysoTracker DND green probe to visualize granules. As shown in Fig. 2.10 A, the upper panels depict granules in green and the middle panels represent mitochondria in red. The lower panels represent images merged from the two previous panels. At resting state, mitochondria inside LAD2 cells are gathered around the nucleus (Fig. 2.10 A, far left panels). After SP stimulation (2 μM, 30 min), mitochondria translocate to the cell membrane region (Fig. 2.10 A, middle left panels, indicated with white arrows). Preincubation with quercetin (100 μM, 30 min) barely has any effect on mitochondrial translocation (Fig. 2.10 A, middle right panels). On the other hand, preincubation with luteolin (100 μM, 30 min) prevents SP-triggered mitochondrial translocation, and the morphological appearance of SP-triggered cells is similar to control cells (Fig. 2.10 A, far right panels).

Fig. 2.10 B represents the percentage of mast cells with mitochondrial translocation obtained from 100 randomly selected cells. Luteolin preincubation (100 μM, 30 min) significantly decreases SP-triggered mitochondrial translocation by approximately 30%.

Figure 2.10. Effects of quercetin and luteolin on SP-triggered mitochondrial translocation in LAD2 mast cells

LAD2 cells were incubated with 20 nM MitoTracker deep red for 20 min and 100 nM LysoTracker DND for 30 min. Cells were washed and incubated with quercetin or luteolin (10-100 µM, 30 min) before SP stimulation (2 µM, 30 min). Cells were observed under Confocal Microscopy. (A) Representative Confocal images of mitochondrial translocation (as indicated by white arrows) in LAD2 cells. Green dye indicates mast cell granules and red dye indicates mitochondria. (B) The percentage of cells with mitochondrial translocation was calculated from 100 randomly selected cells. (Magnification = × 630). Que, quercetin; Lut, luteolin; mt, mitochondrial.

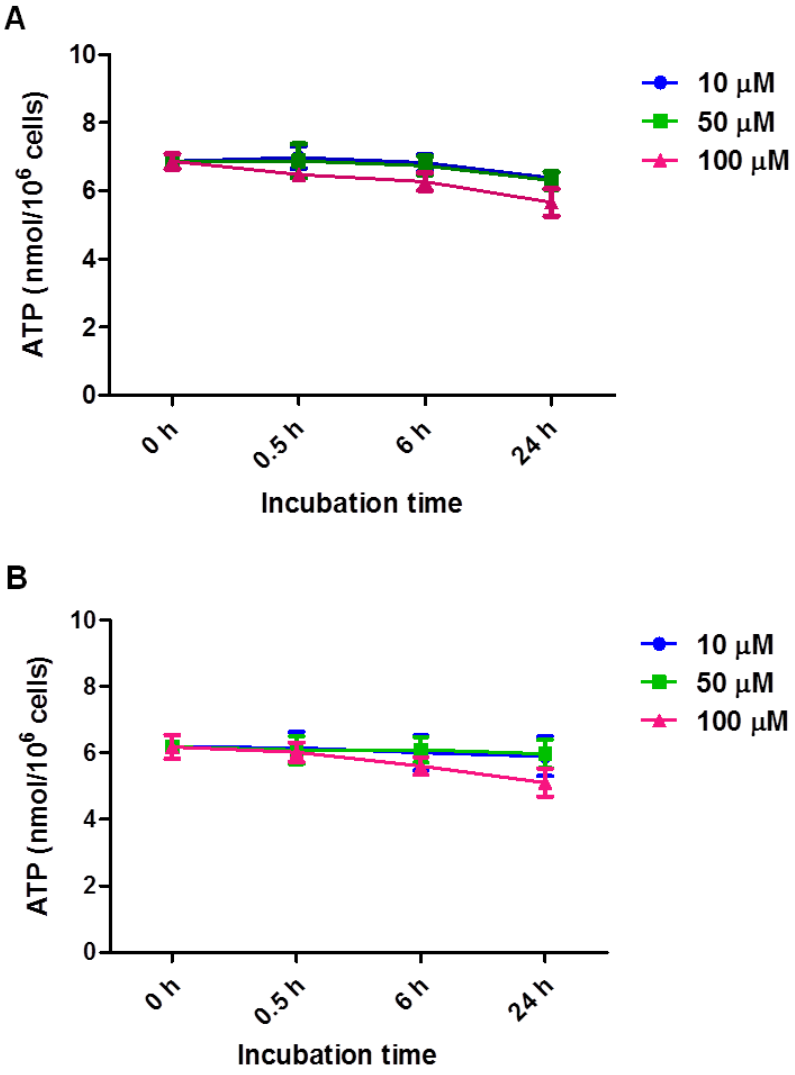


Luteolin and methoxyluteolin do not affect intracellular ATP levels

Mast cell degranulation and protein synthesis require energy. In order to investigate if luteolin and methoxyluteolin have any effect on cellular metabolic activity, we examined the effect of luteolin and methoxyluteolin on intracellular ATP levels in LAD2 cells. SP stimulation does not alter intracellular ATP content in LAD2 cells (data not shown). Luteolin or methoxyluteolin (10 μM and 50 μM) incubation do not alter intracellular ATP content at 24 h (Fig. 2.11). Luteolin at 100 μM for 24 h decreases intracellular ATP content by 10% and methoxyluteolin by 15%, but this apparent decrease is not statistically significant.

Figure 2.11. Effect of luteolin and methoxyluteolin on intracellular ATP levels in LAD2 mast cells

After incubation with different concentrations (10-100 µM) of luteolin (A) or methoxyluteolin (B) for up to 24 h, LAD2 cells (1×10^6) were lysed and intracellular ATP content was determined.

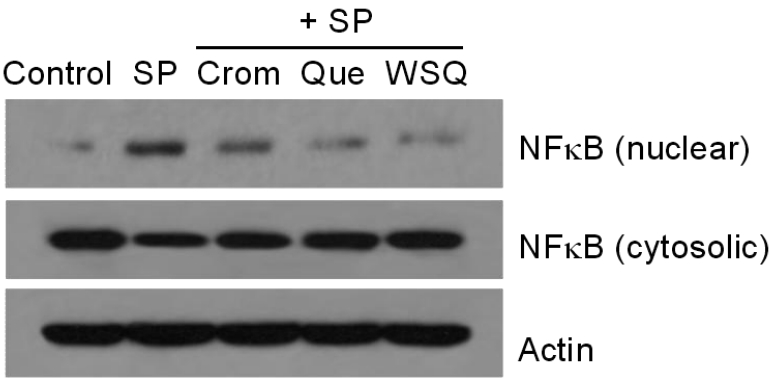


Effect of cromolyn and quercetin on NF-κB nuclear translocation in LAD2 cells

NF-κB is involved in the expression of a number of inflammatory mediators. We investigated whether flavonoids may exert their inhibitory effect on NF-κB activation in human mast cells. We first tested the effect of cromolyn and quercetin on NF-κB nuclear translocation in LAD2 cells. Cytosolic NF-κB protein levels do not change before and after SP stimulation (Fig. 2.12). SP induces NF-κB translocation into the nucleus within 60 min, which is slightly reduced by cromolyn (100 μM, added together with the trigger SP). Preincubation with quercetin and WSQ (100 μM, 6 h) inhibits NF-κB nuclear translocation more effectively than cromolyn as shown by the representative Western blot (Fig. 2.12).

Figure 2.12. Effect of quercetin on nuclear translocation of NF-κB in LAD2 mast cells

LAD2 cells were preincubated with quercetin or WSQ (100 μM, 6 h) before stimulation with SP (2 μM, 60 min). In some experiments, cells were treated with cromolyn (100 μM) and SP (2 μM) at the same time. Upper lane, NF-κB protein levels in nuclear fractions before and after SP stimulation; Middle lane, NF-κB protein levels in cytosolic fractions before and after SP stimulation. Lower lane, β-actin was used as a loading control. Crom, cromolyn; Que, quercetin.

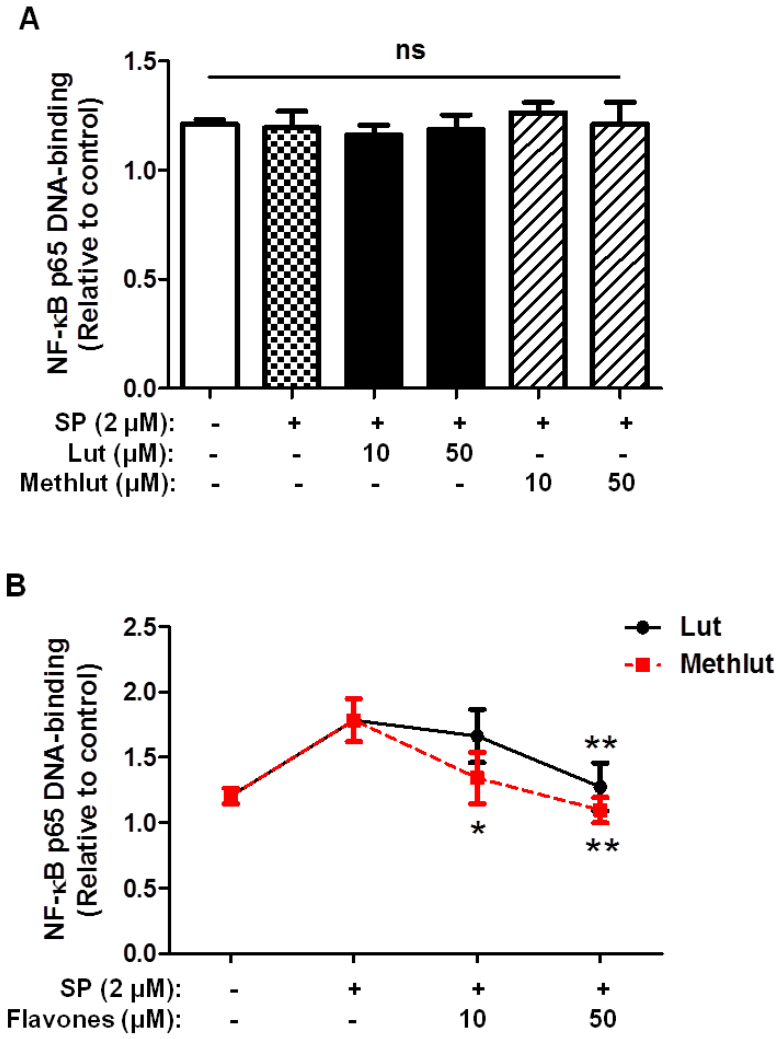


Luteolin and methoxyluteolin reduce NF-κB p65 DNA-binding activity in LAD2 mast cells

We further studied the effects of luteolin and methoxyluteolin on NF-κB activation. In LAD2 cells, SP (2 μM, 15 min) induces NF-κB p65 nuclear translocation and increases the DNA-binding activity of NF-κB p65 inside the nucleus (Fig. 2.13 B). Preincubation with 10 μM of methoxyluteolin (6 h) decreases NF-κB p65 DNA-binding activity in the nucleus by more than 50% compared to control cells, whereas preincubation with 10 μM of luteolin (6 h) has no effect. Preincubation with 50 μM of luteolin or methoxyluteolin (6 h) completely blocks the NF-κB p65 DNA-binding activities in the nucleus (Fig. 2.13 B).

Figure 2.13. Effect of luteolin and methoxyluteolin on NF-κB DNA-binding activity in LAD2 mast cells

LAD2 cells were incubated with luteolin or methoxyluteolin (10-100 μM, 6 h) before stimulation with SP (2 μM, 15 min). Cytosolic and nuclear fractions were separated. NF-κB p65 DNA-binding activities in the (A) cytosolic and (B) nuclear fractions were measured by ELISA and normalized as fold changes relative to control. * p<0.05, **p<0.01, ns=not significant. Lut, luteolin; Methlut, methoxyluteolin.



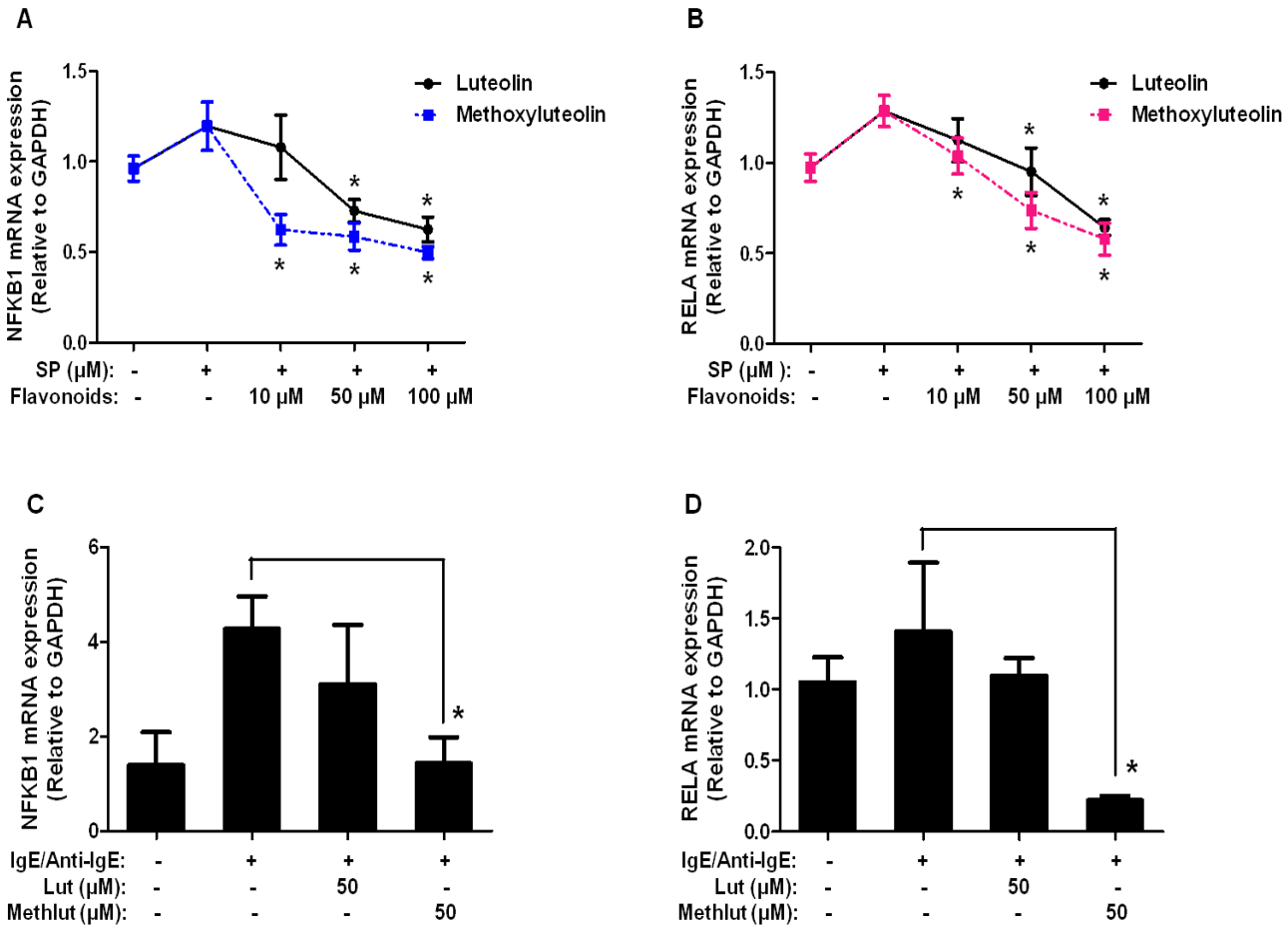
Luteolin and methoxyluteolin decrease mRNA expression of NF-κB-related genes

The mRNA expression of genes encoding two subunits in the NF-κB protein complex was determined by quantitative RT-PCR. NFKB1 encodes for NF-κB p50 subunit and RELA encodes for NF-κB p65 subunit. In LAD2 cells, SP (2 μM, 6 h) slightly induces mRNA expression levels of NFKB1 (Fig. 2.14 A) and RELA (Fig. 2.14 B), which decrease after luteolin or methoxyluteolin preincubation (10-100 μM, 30 min). The inhibitory effect of methoxyluteolin is greater than that of luteolin, especially in inhibiting NFKB1 mRNA expression.

Similar results are seen in primary hCBMCs, where preincubation with methoxyluteolin (50 μM, 30 min) significantly decreases mRNA expression levels of both NFKB1 (Fig. 2.14 C) and RELA (Fig. 2.14 D). Preincubation with luteolin (50 μM, 30 min) slightly reduces the mRNA expression of NFKB1 and RELA, however, this effect is not statistically significant.

Figure 2.14. Effect of luteolin and methoxyluteolin on mRNA expression of NF-κB-related genes

LAD2 cells (A, B) were incubated with luteolin or methoxyluteolin (10-100 μM) for 30 min before stimulation with SP (2 μM, 6 h). hCBMCs (C, D) were first primed overnight with IgE (1 μg/mL), and then incubated with luteolin or methoxyluteolin (50 μM, 30 min) before stimulation with anti-IgE (10 μg/mL, 6 h). Total RNA was isolated and mRNA expression levels of NFKB1 (A, C) and RELA (B, D) were examined by quantitative RT-PCR. *p<0.05. Lut, luteolin; Methlut, methoxyluteolin.



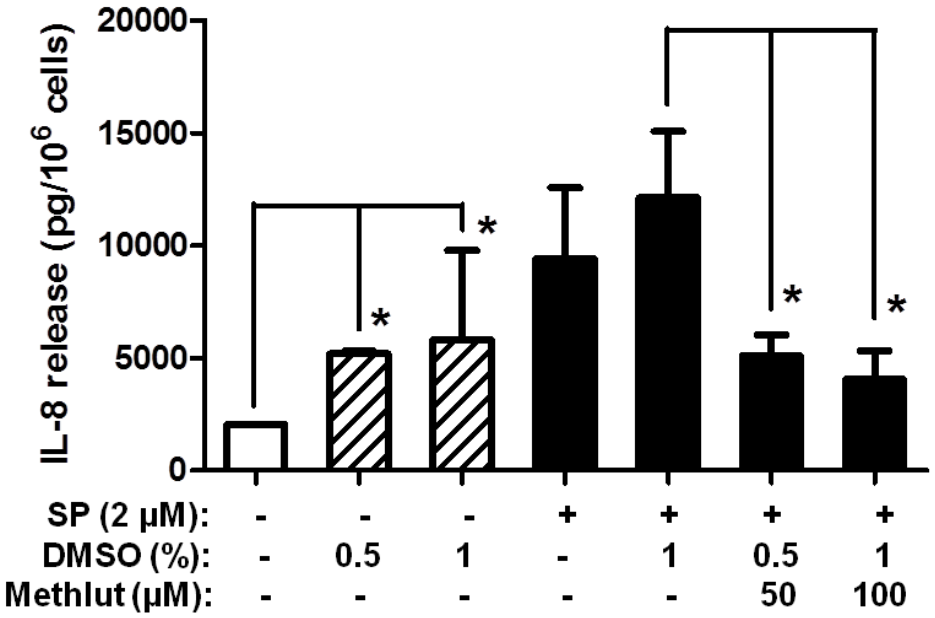
DMSO induces IL-8 release from LAD2 mast cells

Luteolin and methoxyluteolin were prepared in DMSO. To determine the effect of DMSO on mast cells, we examined LAD2 mast cell mediator release in response to DMSO. The final DMSO concentration in culture media was 0.1% for luteolin and 1% for methoxyluteolin at their highest concentration tested (100 μM). DMSO at 0.1% has no effect on mast cells (data not shown). DMSO (0.5% and 1%) significantly increases IL-8 release by more than one-fold in LAD2 mast cells. DMSO also augments SP-induced IL-8 release by about 15% (Fig. 2.15), althought this effect was not statistically significant.

Preincubation with methoxyluteolin (50 and 100 μM, 30 min) significantly inhibits SP-indcued IL-8 release, even in the presence of 0.5% and 1% of DMSO vehicle (Fig. 2.15).

Figure 2.15. Effect of methoxyluteolin on DMSO-induced IL-8 release in LAD2 mast cells

LAD2 cells were incubated with methoxyluteolin (50 and 100 μM, 30 min) before stimulation with SP (2 μM, 24 h). Vehicle control cells were treated with DMSO (1%, 30 min) before the same stimulation with SP (2 μM, 24 h). Some cells were treated with 0.5% and 1% of DMSO alone (24 h). IL-8 release into the supernatant fluids was assayed by ELISA. *p<0.05. Methlut, methoxyluteolin.

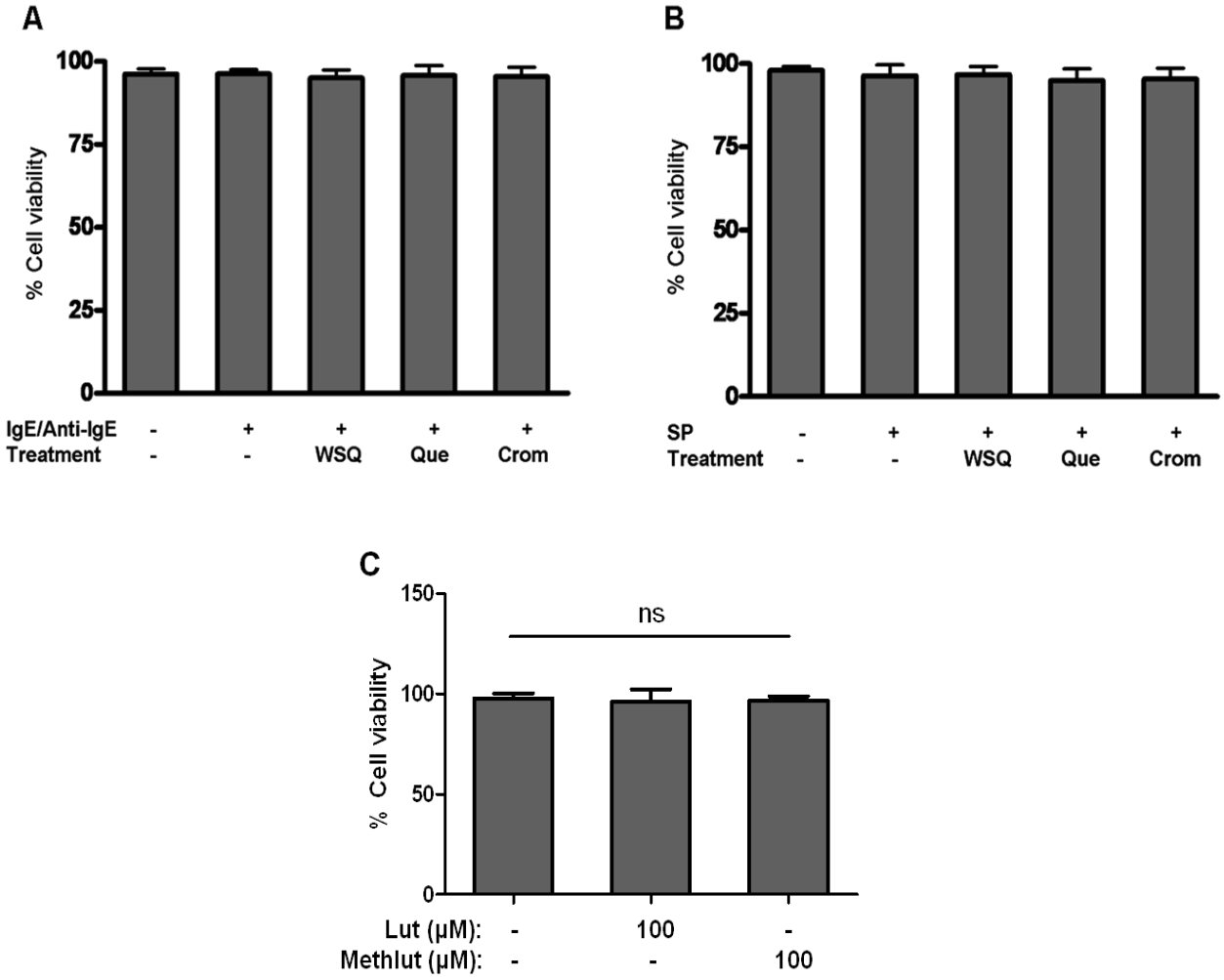


Flavonoids do not alter mast cell viability

After incubation with various flavonoids and cromolyn (100 µM, 24 h), cell viability was determined in primary hCBMCs (Fig. 2.16 A), and LAD2 mast cells (2.16 B, C). Cromolyn, quercetin, WSQ, luteolin and methoxyluteolin have no effect on cell viability after incubation. Both primary hCBMCs and LAD2 mast cells remained >95% viable.

Figure 2.16. Effect of flavonoids on mast cell viability

Primary hCBMCs (A) and LAD2 cells (B, C) were incubated with various flavonoids and cromolyn (100 µM) for 24 h. Cell viability was determined by Trypan blue (0.4%) exclusion test (ns, not statistically significant). ns=not significant. WSQ, water-soluble quercetin; Que, quercetin; Crom, cromolyn; Lut, luteolin; Methlut, methoxyluteolin.



Discussion

Our results report on the comparative inhibitory effects of the "mast cell stabilizer" cromolyn, and the flavonoids quercetin, WSQ, luteolin and methoxyluteolin on human mast cell inflammatory mediator secretion. We also show the effects of these flavonoids on intracellular calcium ion levels, mitochondrial translocation and activation of the nuclear factor NF-κB. Methoxyluteolin is the most effective inhibitor of human mast cell activation, followed by luteolin, WSQ and then quercetin, where cromolyn has the least inhibitory action. WSQ is a quercetin chalcone structurally derived from quercetin, differing in that the center ring is open and the oxygen atom in the center ring is converted to a hydroxyl group (Fig. 1.1 F); at least, the 5,7-hydroxyl groups are in their sodium salt. WSQ is more water soluble than quercetin probably due to the additional phenolic hydroxyl group. This group could also enhance its antioxidant property. Cromolyn partially inhibits mast cell degranulation, but it has to be added simultaneously with the trigger. Cromolyn also has no effect on delayed cytokine release. The lack of cromolyn's inhibitory action when used prophylactically has been reported previously and termed as tachyphylaxis[202]. Hence, although the structures of these flavonoids have some similarities to that of cromolyn (Fig. 1.1), their actions on human mast cells appear to differ considerably.

Previously, quercetin had been reported to inhibit histamine release from rat connective tissue mast cells and mucosal mast cells[203], as well as from human lung and intestinal mast cells[143]. We recently showed that luteolin inhibits VEGF release from mercury-activated human LAD2 mast cells[190]. Our results here further demonstrate that

these flavonoids broadly inhibit human mast cell degranulation to release multiple preformed mediators, such as histamine, β-hex, LTs, PGD_2 and prestored TNF, as well as delayed secretion of newly-synthesized cytokines, chemokines and growth factors, including TNF, IL-6, IL-8, CCL2 and VEGF. In addition, these flavonoids are equally effective against mast cell activation stimulated by different triggers, including the allergic trigger IgE/anti-IgE, the cytokine IL-33, and the neuropeptide SP. Moreover, the fact that quercetin, luteolin and methoxyluteolin decrease gene expression of inflammatory mediators is significant, indicating their ability to inhibit mast cell mediator production before they are released from the cells.

To determine the mechanim of action, we first studied the effects of these flavonoids on intracellular calcium elevation, which is necessary for human mast cell mediator release[204]. Both luteolin and methoxyluteolin is more effective than quercetin in blocking intracellular calcium increase in stimulated human mast cells, while cromolyn has no effect. Previous studies had reported that activation of hCBMCs by IgE/anti-IgE increased intracellular calcium levels[197, 205]. Fewtrell and Gomperts had first shown that quercetin could inhibit calcium influx in rat peritoneal mast cells[187]. Previous reports also showed that IgE-mediated release of histamine and LTs was abolished after calcium depletion[206]. Quercetin also inhibited ionophore-induced histamine release from rat peritoneal mast cells, suggesting it had actions other than receptor-mediated calcium influx[193]. Because methoxyluteolin has four methoxyl groups substituted for the four hydroxyl groups on luteolin (Fig. 1.1 C, D), the calcium-blocking actions of quercetin and luteolin are unlikely to be due to their poly-phenolic structures, where calcium-

chelation could occur. Instead, these flavonoids may inhibit or activate the regulatory components of calcium signaling pathways in mast cells. In fact, luteolin-7-O-glucoside isolated from Ailanthus altissima has been shown to inhibit phospholipase C phosphorylation in mBMMCs[207].

We also demonstrate for the first time to our knowledge, that luteolin reduces mitochondrial translocation, a critical process accompanying mast cell degranulation[208]. However, the extent of inhibition on mitochondrial translocation (30% reduction) does not correlate with the extent of inhibition on degranulation (60% decreases). This suggests that decreasing mitochondrial translocation is only one part of the inhibitory actions of luteolin on mast cell degranulation.

Interestingly, luteolin is readily absorbed by mast cells and becomes associated with mitochondria (data presented in Chapter 4). Mitochondria are the main site for the production of ATP, which is absolutely required as the energy source for mast cell mediator release. Intriguingly, luteolin and methoxyluteolin treatment only slightly reduced about 10% of intracellular ATP content in LAD mast cells, which is not likely to account for their strong inhibitory actions on mast cell degranulation. Nevertheless, luteolin and methoxyluteolin may reduce or antagonize ATP utilization more potently at specific sites inside the cell during the secretion process.

We further studied the effects of flavonoids on the inducible transcription factor NF-κB, which is a protein complex that translocates from the cytoplasm into the nucleus

once activated and regulates gene expression of various inflammatory molecules and cytokines, such as TNF, IL-6 and IL-8[209]. Firstly, we show that the flavonol quercetin effectively blocks nuclear translocation of NF-κB p65, where cromolyn has minimal effect. We then show that luteolin and methoxyluteolin decrease nuclear translocation and DNA-binding activity of NF-κB p65. Two previous studies had reported that quercetin [189] and luteolin [70] inhibit inflammatory cytokine production by blocking NF-κB pathway in the HMC-1 cell line. However, the HMC-1 cell line represents an immature leukemic human mast cell, which does not express the high affinity FcεRI receptor for IgE[210]. HMC-1 cells also proliferates independent of SCF, which is absolutely required for proliferation of primary human mast cells in culture. In our studies, we used LAD2 mast cells, which represent more mature human mast cells than HMC-1 cells with functional FcεRI receptors and the dependence on SCF for growth[196]. Our results confirm that quercetin and luteolin reduce inflammatory mediator production by inhibiting NF-κB activation in human mast cells.

In addition, the fact that luteolin and methoxyluteolin decrease NF-κB p65 DNA-binding activity inside the nucleus suggests that they pass the plasma membrane and enter the nucleus in sufficient amounts. A previous publication had reported the ability of quercetin to actively accumulate in the nucleus and modify the activity of numerous transcription factors[211]. As structurally-related flavones, luteolin and methoxyluteolin might have similar actions and regulate gene transcription inside the nucleus.

Apart from inhibiting NF-κB activation at the protein level, we also report for the first time to our knowledge that luteolin and methoxyluteolin decrease mRNA expression of genes encoding two different subunits in the NF-κB protein complex, NFKB1 (encoding NF-κB p50 subunit) and RELA (encoding NF-κB p65 subunit). By blocking NF-κB activation at both the gene and protein expression levels, luteolin and methoxyluteolin can effectively regulate proinflammatory mediator production.

As seen from our results, the extent of inhibition of mast cell activation is greatest for methoxyluteolin, followed by luteolin and then quercetin. The molecular structures of luteolin and quercetin only differ by one hydroxyl group substitution on the C3 position of the middle ring (Fig. 1.1 C, D), yet luteolin is more effective than quercetin in decreasing mediator release. A previous study comparing antiplatelet effects of flavonoids showed that flavones (luteolin and apigenin) have significantly higher affinity for the thromboxane A2 receptor than flavonols (quercetin) that have a substitution in C3 position[212]. This result is in accordance with our finding, and suggests that the mast cell-inhibitory and antiplatelet actions might depend on a similar chemical structure that is shared between flavones. Another possible explanation is that luteolin penetrates cells better than quercetin, which is presented and discussed in Chapter 4 (Table 4.1).

The substitution of 3', 4', 5, 7-tetrahydroxyl groups by four methoxyl groups (methoxyluteolin, Fig. 1.1 E) further enhances the inhibitory activity of luteolin. This finding seems to contradict previous studies on the structure-activity relationship of flavonoids, which showed that flavones containing more hydroxyl groups have greater

anti-asthmatic effects[213]. Moreover, hydroxylation of positions 7 and 4' is essential for radical-scavenging capacity that contributes to the antioxidant actions of flavonoids[214]. However, a study by Tominari *et al* has demonstrated that two polymethoxy flavonoids (nobiletin (3',4',5,6,7,8-hexamethoxy flavone) and tangeretin (4',5,6,7,8-pentamethoxy flavone)) prevent LPS-induced bone loss in a mouse model for periodontitis and inhibit PGE2 production in co-cultures of bone marrow cells and osteoblasts[215]. A recent paper also showed that 6-methoxyluteolin potently blocks histamine release and intracellular calcium elevation in human basophilic KU812F cells[216]. Moreover, 5-methoxyquercetin, 7-methoxyquercetin and 5,7-dimethoxyquercetin have stronger thrombin inhibition activity than quercetin, indicating that 5-hydroxyl group and 7-hydroxyl group in the A ring can be modified without reducing the anticoagulant activity of quercetin[217]. These studies support our findings and suggest that flavonoids can interfere with cellular processes using specific mechanisms other than the well-referenced and widely (sometimes inaccurately) acclaimed antioxidant properties, which require multiple hydroxyl groups that are absent in methoxyluteolin and other polymethoxy flavonoids. In addition, the less polar structure of polymethoxy flavonoids enhances their permeability into cellular membranes and binding properties, thereby increasing their biological activities.

More interestingly, Wen and Walle in 2006 reported that methylated flavones have high resistance to hepatic metabolism and have greatly enhanced intestinal absorption compared to their corresponding unmethylated flavones, as well as resveratrol and quercetin, which are rapidly eliminated due to extensive glucuronidation and/or

sulfation[218]. This finding is of particular importance because human intestinal UGTs and sulfotransferases effectively conjugate the 3'- and 4'-hydroxyl moieties of quercetin and luteolin[219], resulting in low oral bioavailability. The improved metabolic stability and intestinal absorption of the methylated flavones may contribute to their enhanced pharmacological actions.

In conclusion, our results indicate that quercetin is more effective than cromolyn in inhibiting human mast cell activation. Both luteolin and methoxyluteolin is even more potent than quercetin in blocking human mast cell degranulation and inflammatory mediator release. The likely mechanisms of action of these flavonoids involve decreasing intracellular calcium levels, reducing mitochondrial translocation, and decreasing NF-κB activation at both the transcriptional and translational levels. Luteolin and methoxyluteolin have the potential to be developed into promising mast cell stabilizers, particularly when made into formulations that increase their solubility and absorption.

Chapter 3. Luteolin and Methoxyluteolin Inhibit Human Keratinocyte Activation

Introduction

Keratinocytes are fully immunocompetent and can release inflammatory mediators such as IL-1, IL-6, IL-8, and VEGF, in response to immune and endocrine triggers[71-73]. Keratinocytes are involved in the pathogenesis of Ps, through their hyperproliferation and associated chronic inflammation[77-79]. With a prevalence of approximately 2-3% of the world's population and typical lifetime duration of more than 30 years, the combined cost of Ps has a major impact on health care.

TNF plays a key role in the pathogenesis of Ps [102] and is produced by a number of cell types, including activated keratinocytes, T cells and mast cells[80]. TNF can trigger keratinocytes to produce IL-1[103], which then stimulates both keratinocytes and mast cells to release IL-6[32]. IL-6 then exerts autocrine actions and stimulates proliferation of cultured human keratinocytes[104]. TNF stimulates keratinocytes to release VEGF, which leads to dysregulated angiogenesis that contributes to the development of Ps-like dermatitis[72, 105]. In addition, TNF triggers activation of nuclear factor-kappa B (NF-κB), a transcription factor involved in inflammatory mediator production, which is upregulated in Ps lesional skin[106].

Since the role of TNF in Ps pathogenesis has become more recognized, anti-TNF biologic agents, such as etanercept, have been developed and shown to be more effective than traditional treatments for Ps, including methotrexate, psoralen plus ultraviolet A (PUVA), cyclosporine, and acitretin[80, 124]. Unfortunately, anti-TNF agents are also associated with increased risk of infections, and more recently blood malignancies[127, 128]. Other biologic agents have been introduced targeting IL-12, IL-17 and IL-23[129, 130], but the long-term efficacy data remain unknown[131]. Therefore, the need for safe and effective long-term treatments for Ps is still of major importance.

Recently an increasing number of plant-derived molecules have shown positive effects in clinical trials in the treatment of inflammatory skin diseases[220], but the specific ingredients in the plant extracts are not well defined. Flavonoids, such as quercetin and luteolin, are natural compounds found in a number of plants with potent anti-oxidant and anti-inflammatory actions[147, 221]. The flavonol quercetin suppresses ultraviolet irradiation-induced expression of inflammatory cytokines IL-1β, IL-6, IL-8 and TNF in human keratinocytes[222]. Quercetin has also been shown to inhibit contact dermatitis and photosensitivity in humans[223]. However, the precise mechanisms of action of flavonoids on human keratinocytes are not fully understood.

In the previous chapter, we show that the two flavones, luteolin and methoxyluteolin, are more effective than the flavonol quercetin in inhibiting activation of human mast cells. In this chapter, we further studied effects of these two flavones on

inhibition of inflammatory mediator release and proliferation of human cultured keratinocytes.

Methods

Drugs and Reagents

Luteolin was purchased from Sigma-Aldrich (St. Louis, MO). Methoxyluteolin was obtained from Pharmascience Nutrients (Clear Water, FL). The two flavones were dissolved in DMSO. Recombinant human TNF and IL-17 were purchased from R&D Systems (Minneapolis, MN) and dissolved in distilled water. The final concentration of DMSO was < 0.1% and the pH was 7.4. Rabbit monoclonal antibodies against NF-κB p65 (D14E12) and β-actin (D6A8), and mouse monoclonal antibody against lamin A/C (4C11) were purchased from Cell Signaling (Danvers, MA).

Human keratinocytes

The immortalized human keratinocyte cell line HaCaT was used since it has been shown to be suitable for studies relavant to Ps[224, 225]. HaCaT keratinocytes were kindly provided by Dr. A. Slominski (University of Tennessee, Menphis, TN) and were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma-Aldrich).

Adult normal human epidermal keratinocytes (NHEKs) purchased from Life Technologies (Carlsbad, CA) were cultured in EpiLife serum-free medium containing Human Keratinocyte Growth Supplement (HKGS, Life Technologies).

Cytokine release by Enzyme-Linked Immunosorbent Assay (ELISA)

Keratinocytes (5×10^4 cells per well) were seeded in 12-well plates (Becton Dickinson, Franklin Lakes, NJ) and allowed to grow overnight before stimulation with either TNF or IL-17 (50 ng/mL, 24 h). In some experiments, cells were pretreated with luteolin or methoxyluteolin (1-100 μM for up to 24 h), while control cells were treated with DMSO (0.1%). IL-6, IL-8 and VEGF were measured in supernatant fluids by ELISA using commercial kits from R&D Systems.

Human subjects

Punch skin biopsies (3 mm^3) were collected from non-exposed lesional skin (back and gluteal) from Ps patients, who had not received any medication for 15 days prior to biopsy, and were free from any systemic allergic or inflammatory diseases, as well as healthy controls (n=26-30). Biopsies were immediately placed in RNAlater solutions (Ambion, Inc., Austin, Texas) and stored at -80˚C. The Medical Ethics Committees of Attikon and A. Sygros Hospitals approved this protocol. The Declaration of Helsinki protocols were followed and patients provided their written, informed consent. All human samples had no identifiers except for age and sex.

RNA isolation and quantitative real time PCR (qRT-PCR)

Keratinocytes were pretreated with luteolin or methoxyluteolin (1-100 μM, 30 min) before stimulation with TNF or IL-17 (50 ng/mL, 6 h). Total RNA was extracted with an RNeasy Mini kit (Qiagen Inc., Valencia, CA). For human skin samples, total RNA was extracted using a Fibrous Tissue mini kit (Qiagen). An iScript cDNA synthesis

kit (BioRad, Hercules, CA) was used for reverse-transcription of each sample. qRT-PCR was performed using Taqman gene expression assays (Applied Biosystems, Foster City, CA) for IL-6, IL-8, VEGF, NFKB1, RELA and TNFAIP3. Samples were run using a 7300 Sequence Detector, according to TaqMan Gene Expression Assay instructions (Applied Biosystems). The mRNA expression was determined from standard curves run with each experiment. Relative mRNA levels were normalized to GAPDH endogenous control (Applied Biosystems).

NF-κB p65 phosphorylation assay

After preincubation with luteolin or methoxyluteolin (10-100 μM, 6 h), HaCaT cells (2×10^6 cells) were stimulated with TNF (50 ng/mL, 15 min). Phosphorylation of NF-κB p65 (serine 536) was detected by the PathScan Inflammation Sandwich ELISA kit (#7276, Cell Signaling) according to the instructions provided. Whole cell lysates were assayed at a protein concentration of 5 mg/mL. Absorbance was read at 450 nm using a LabSystems Multiskan RC microplate reader (Fisher Scientific, Cambridge, MA). Relative phospho-NF-κB p65 levels were normalized to control cells treated with DMSO (0.1%).

Nuclear translocation of NF-κB p65 by Western blot analysis

After preincubation with luteolin (100 μM, 6 h), HaCaT cells (2×10^6 cells) were stimulated with TNF (50 ng/mL, 15 min). Cells were harvested and cytosolic and nuclear extracts were isolated using a NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, Rockford, IL). Antibody against the nuclear protein lamin A/C was used to

assess purity of cytosolic and nuclear extracts[226]. Protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Aliquots of the protein extracts (each containing 20 μg of protein) were boiled for 5 min and electrophoresed on NuPAGE 4-12% Bis-Tris gel (Life Technologies). The resolved proteins were then transferred to PVDF membranes before antibody probing at room temperature using a SNAP i.d. system (Millipore, Billerica, MA). Antibody binding was detected using an Amersham ECL Prime detection kit (GE Healthcare, Buckinghamshire, UK). Densitometric analysis of Western blot bands was performed using Image J software downloaded from the NIH website (Bethesda, MD).

NF-κB p65 DNA-binding activity assay

HaCaT cells (10×10^6 cells) were preincubated with luteolin or methoxyluteolin (10-100 μM, 6 h) before stimulation with TNF (50 ng/mL, 15 min). Cells were then harvested and cytosolic and nuclear extracts were isolated as described above. DNA-binding activity of NF-κB p65 was detected by the NF-κB (p65) Transcription Factor Assay Kit (#10007889, Cayman Chemical Co., Ann Arbor, MI). Cytosolic and nuclear extracts (each containing 10 μg of protein) were added to a 96-well plate coated with a specific double stranded DNA sequence containing the NF-κB response element. Relative NF-κB p65 DNA-binding activities were normalized to control cells treated with 0.1% DMSO.

Cell proliferation

HaCaT cells (2×10^5 cells per well) were seeded in 6-well plates and treated with either luteolin or methoxyluteolin (10-100 μM) for up to 3 days. Cell proliferation was measured using the XTT (2, 3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)-based *in vitro* toxicology assay kit (Sigma-Aldrich). Absorbance at 450 nm is directly proportional to the number of live cells. Cell viability was determined by Trypan-blue (0.4%) exclusion.

Intracellular ATP measurement

Intracellular ATP content was measured to determine if luteolin has any effect on cellular energy production. After luteolin incubation (10-100 μM) for 3 days, HaCaT cells (1×10^6) were lysed and intracellular ATP content was determined using an ATP assay kit (Abcam, Cambridge, MA).

Statistical analysis

All experiments were performed in triplicate and were repeated at least three times (n=3). Results are presented as mean ± SD. For *in vitro* experiments, data from stimulated and control samples were compared using the unpaired, two-tailed, Student's *t*-test. For human skin gene expression of NFKB1, RELA and TNFAIP3, results from Ps patients and controls were compared using the Mann-Whitney non parametric U-test. Statistical significance was set at $p < 0.05$.

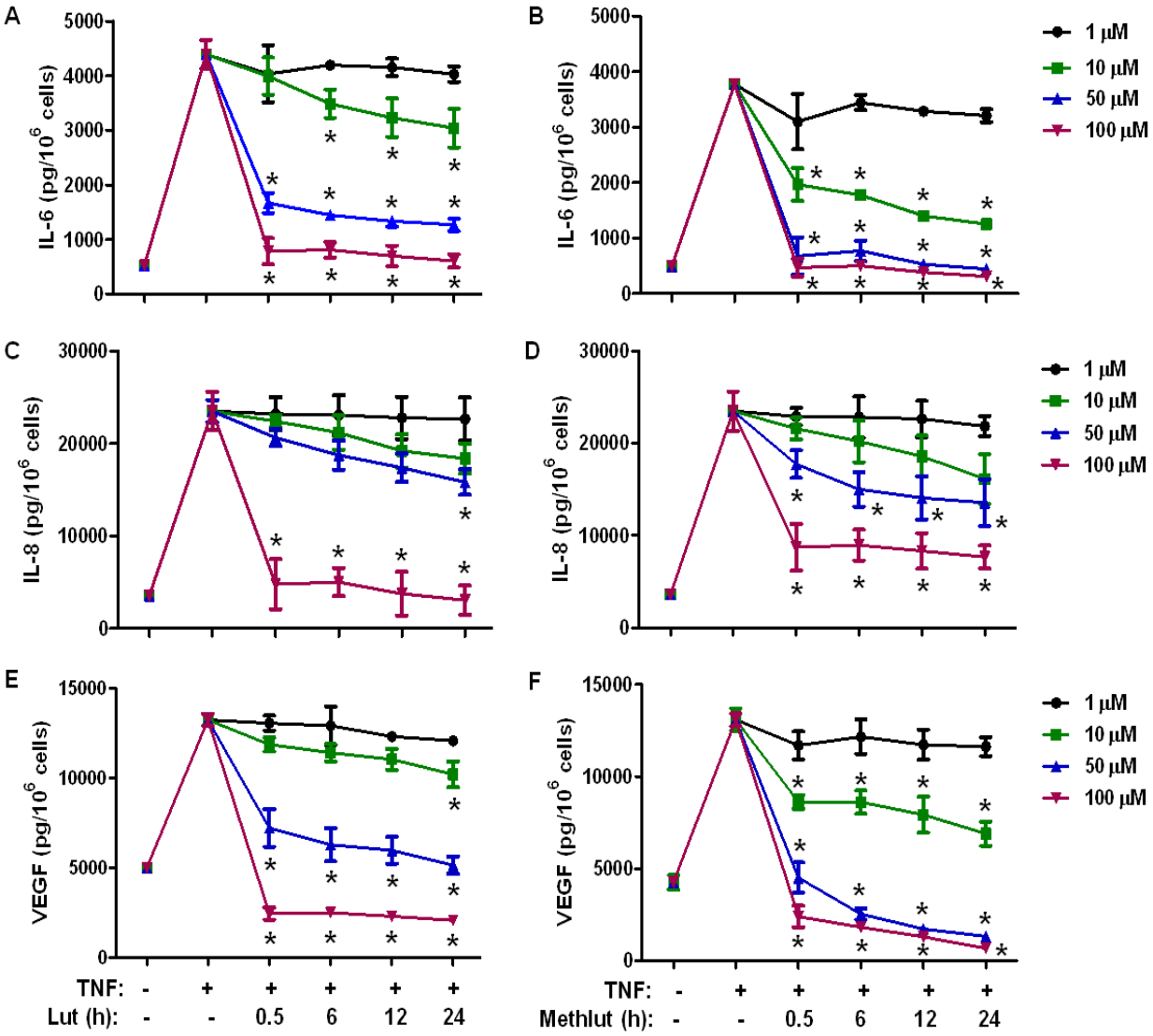
Results

Luteolin and methoxyluteolin inhibit TNF-triggered IL-6, IL-8 and VEGF release from HaCaT keratinocytes

Incubation of HaCaT cells with TNF (50 ng/mL, 24 h) significantly increases IL-6, IL-8 and VEGF release compared to control cells (Fig. 3.1). Preincubation with luteolin and methoxyluteolin significantly inhibits mediator release in a concentration-dependent manner. Methoxyluteolin (10 μM, 30 min) significantly inhibits TNF-induced IL-6 (Fig. 3.1 B) and VEGF release (Fig. 3.1 F), while luteolin at the same concentration has no effect (Fig. 3.1 A, E). Prolonged preincubation does not increase the extent of inhibition, except for IL-6 release where inhibition by luteolin (10 μM) becomes more prominent with preincubation time of 12-24 h as compared to 30 min (Fig. 3.1 A). At higher concentrations (50 and 100 μM) of luteolin and methoxyluteolin, IL-6, IL-8 and VEGF release is almost completely blocked (Fig. 3.1).

Figure 3.1. Effect of luteolin and methoxyluteolin on TNF-triggered IL-6, IL-8 and VEGF release from human HaCaT keratinocytes

HaCaT cells were preincubated with luteolin or methoxyluteolin (1-100 µM) for various times as indicated and then stimulated with TNF (50 ng/mL, 24 h). Mediators in the supernatant fluids were measured by ELISA: (A, B) IL-6; (C, D) IL-8; (E, F) VEGF. Lut, luteolin; Methlut, methoxyluteolin; h, hours. * $p<0.05$.

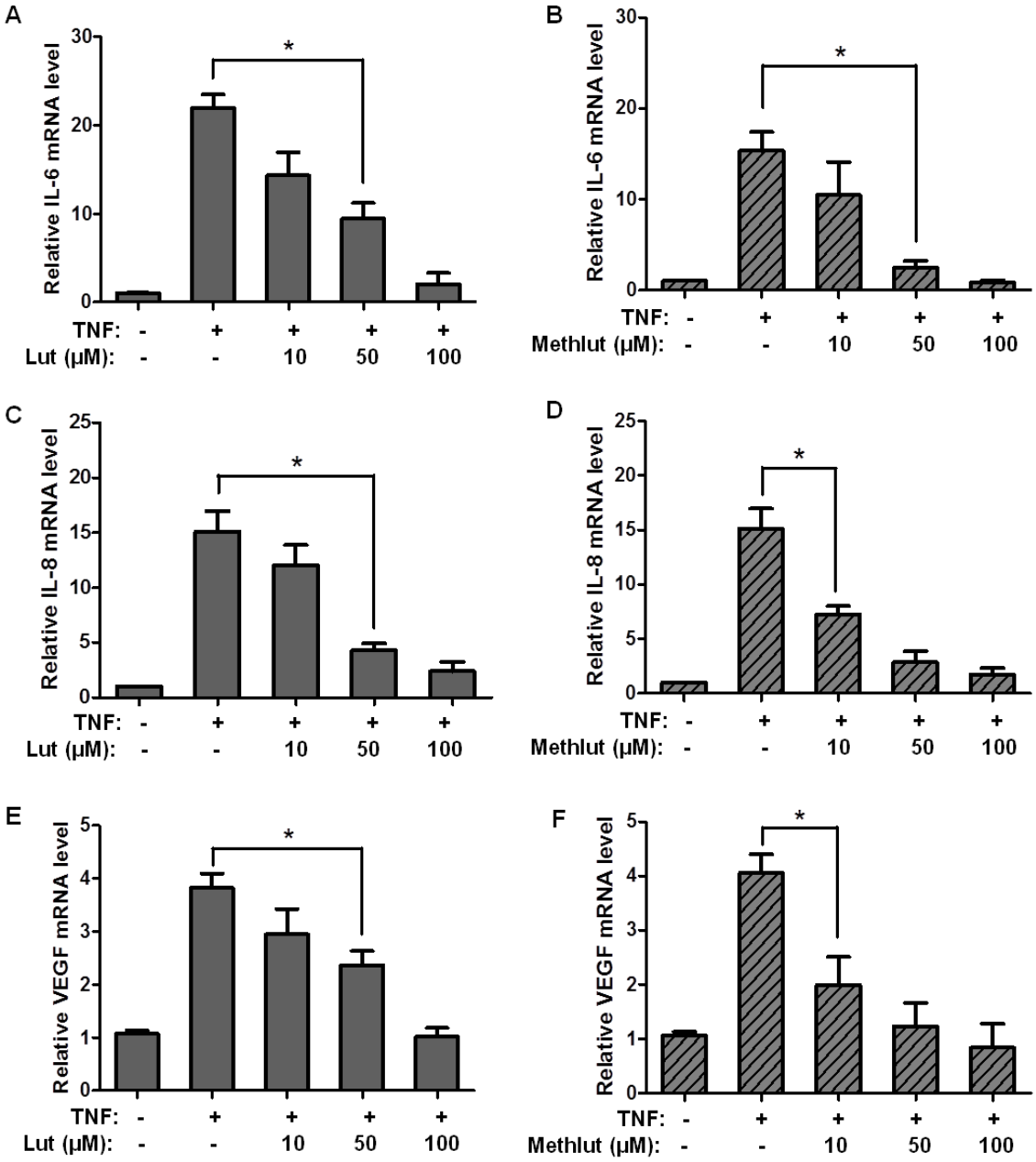


Luteolin and methoxyluteolin inhibit mRNA expression of IL-6, IL-8 and VEGF in TNF-triggered HaCaT keratinocytes

The effect of luteolin and methoxyluteolin was also examined on TNF-triggered mRNA expression of IL-6, IL-8 and VEGF. In HaCaT cells, TNF stimulation (50 ng/mL, 6 h) triggers about a 20-fold increase in IL-6 mRNA level (Fig. 3.2 A, B), a 15-fold increase in IL-8 mRNA level (Fig. 3.2 C, D), and a 4-fold increase in VEGF mRNA level (Fig. 3.2 E, F) as compared to control cells. Preincubation with luteolin or methoxyluteolin (10-100 μM) for 30 min decreases mRNA expression of all three mediators, with complete inhibition achieved at 100 μM. Methoxyluteolin at 10 μM is more potent than luteolin, especially in inhibiting mRNA expression levels of IL-8 (Fig. 3.2 D) and VEGF (Fig. 3.2 F).

Figure 3.2. Effect of luteolin and methoxyluteolin on mRNA expression of IL-6, IL-8 and VEGF in TNF-triggered HaCaT keratinocytes

HaCaT cells were preincubated with luteolin or methoxyluteolin (10-100 μM) for 30 min before stimulation with TNF. The mRNA expression levels of IL-6 (A, B), IL-8 (C, D) and VEGF (E, F) were determined after TNF stimulation (50 ng/mL) for 6 h by qRT-PCR. * $p<0.05$. Lut, luteolin; Methlut, methoxyluteolin.

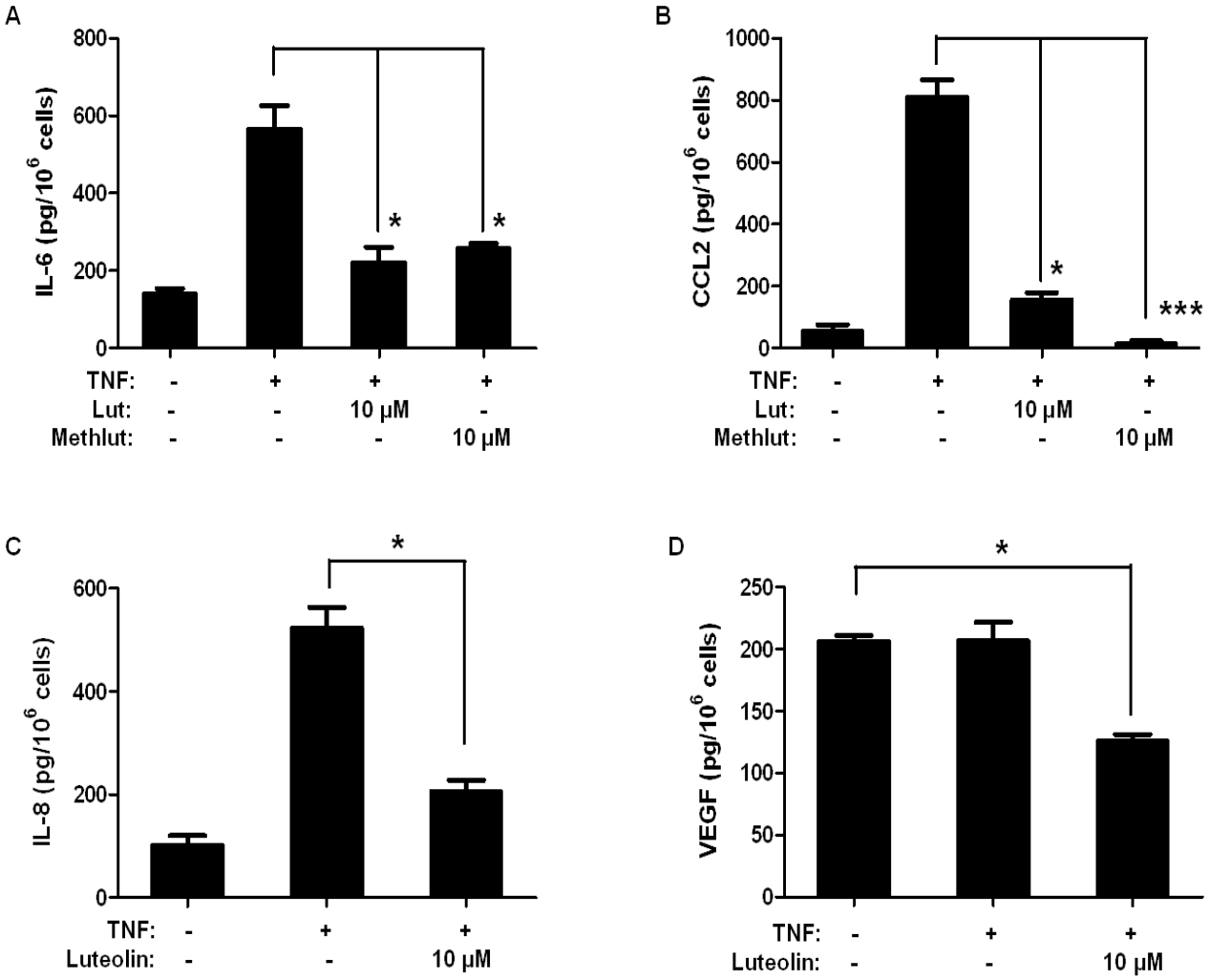


Luteolin and methoxyluteolin decrease TNF-triggered mediator release from primary NHEKs

Apart from HaCaT cells, we also studied the effects of luteolin and methoxyluteolin on mediator release from primary cultured keratinocytes. Stimulation of NHEKs with TNF (50 ng/mL, 24 h) induces IL-6, CCL2 and IL-8 release (Fig. 3.3 A-C), but at a much lower level compared to the amounts of IL-6 and IL-8 secreted by HaCaT cells (Fig. 3.1). TNF does not trigger VEGF release in NHEKs (Fig. 3.3 D) as it does for HaCaT cells. Interestingly, NHEKs are more sensitive than HaCaT cells to the inhibitory effects of luteolin and methoxyluteolin. Preincubation with luteolin (10 μM, 30 min) inhibits TNF-triggered IL-6 and IL-8 release by more than 90% (Fig. 3.3 A, C), and decreases basal VEGF production in NHEKs (Fig. 3.3 D). Preincubation with methoxyluteolin (10 μM, 30 min) inhibits TNF-triggered IL-6 release to a similar extent compared to luteolin (Fig. 3.3 A). Methoxyluteolin is more effective than luteolin in decreasing CCL2 release (100% inhibition, Fig. 3.3 B).

Figure 3.3. Effect of luteolin and methoxyluteolin on TNF-triggered mediator release from NHEKs

NHEKs were preincubated with luteolin or methoxyluteolin (10 µM) for 30 min before stimulation with TNF (50 ng/mL, 24 h). The release of (A) IL-6, (B) CCL2, (C) IL-8 and (D) VEGF were measured by ELISA. * $p<0.05$, *** $p<0.001$. Lut, luteolin; Methlut, methoxyluteolin.

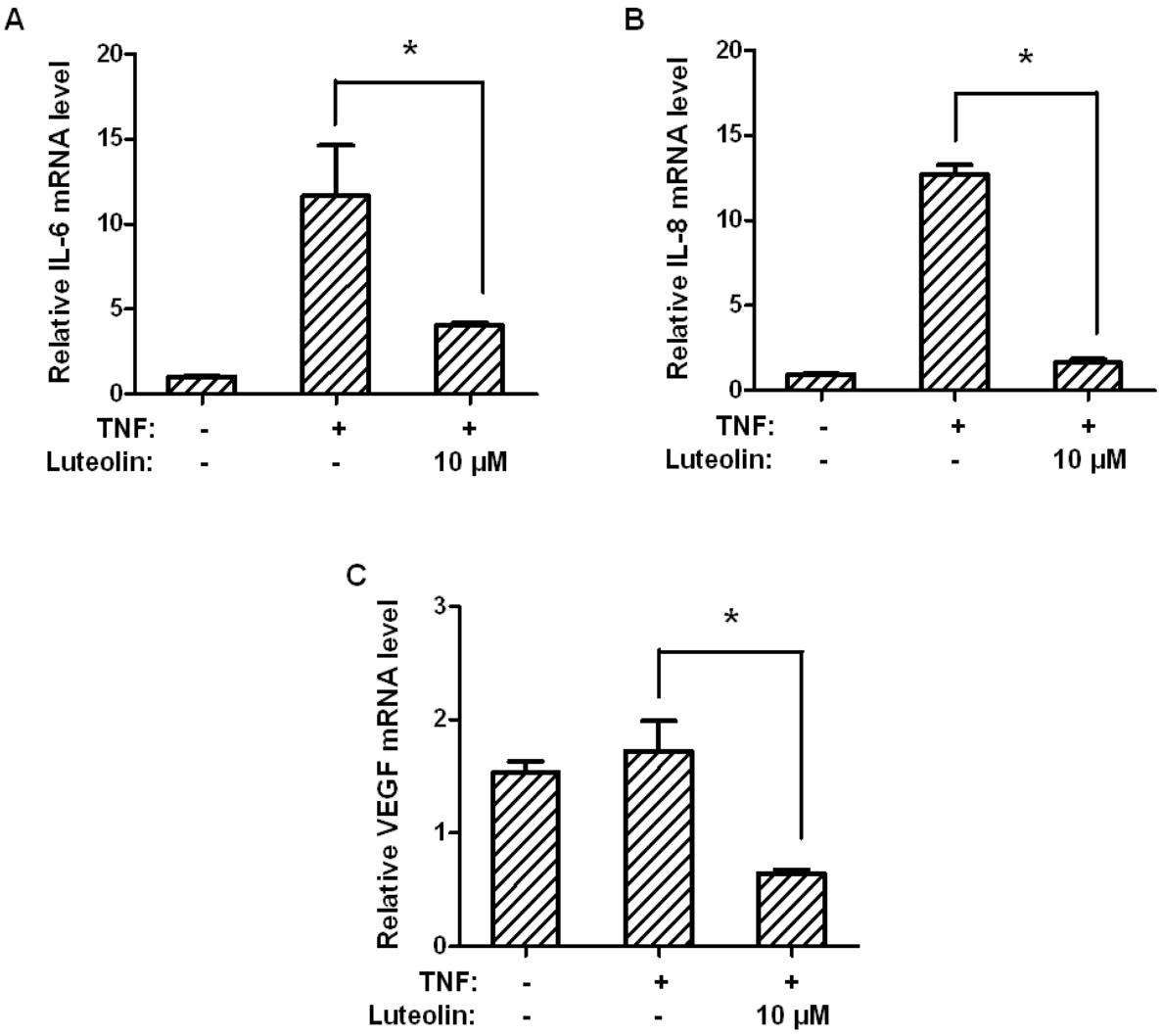


Luteolin inhibits mRNA expression of IL-6, IL-8 and VEGF in TNF-triggered NHEKs

In NHEKs, TNF stimulation (50 ng/mL, 6 h) triggers about 12-fold increase in mRNA levels of IL-6 (Fig. 3.4 A) and IL-8 (Fig. 3.4 B) as compared to control cells. TNF does not stimulate VEGF mRNA expression in NHEKs (Fig. 3.4 C). The mRNA expression of all three mediators is significantly decreased by preincubation with a lower concentration of luteolin (10 µM, 30 min) (Fig. 3.4) as compared to the concentration required (50 µM, 30 min) to achieve similar inhibition in HaCaT cells (Fig. 3.2 A, C, E).

Figure 3.4. Effect of luteolin on mRNA expression of IL-6, IL-8 and VEGF in TNF-triggered NHEKs

NHEKs were preincubated with luteolin (10 μM) for 30 min before stimulation with TNF. The mRNA expression level of (A) IL-6, (B) IL-8 and (C) VEGF was determined after TNF stimulation (50 ng/mL) for 6 h by qRT-PCR. * $p<0.05$.

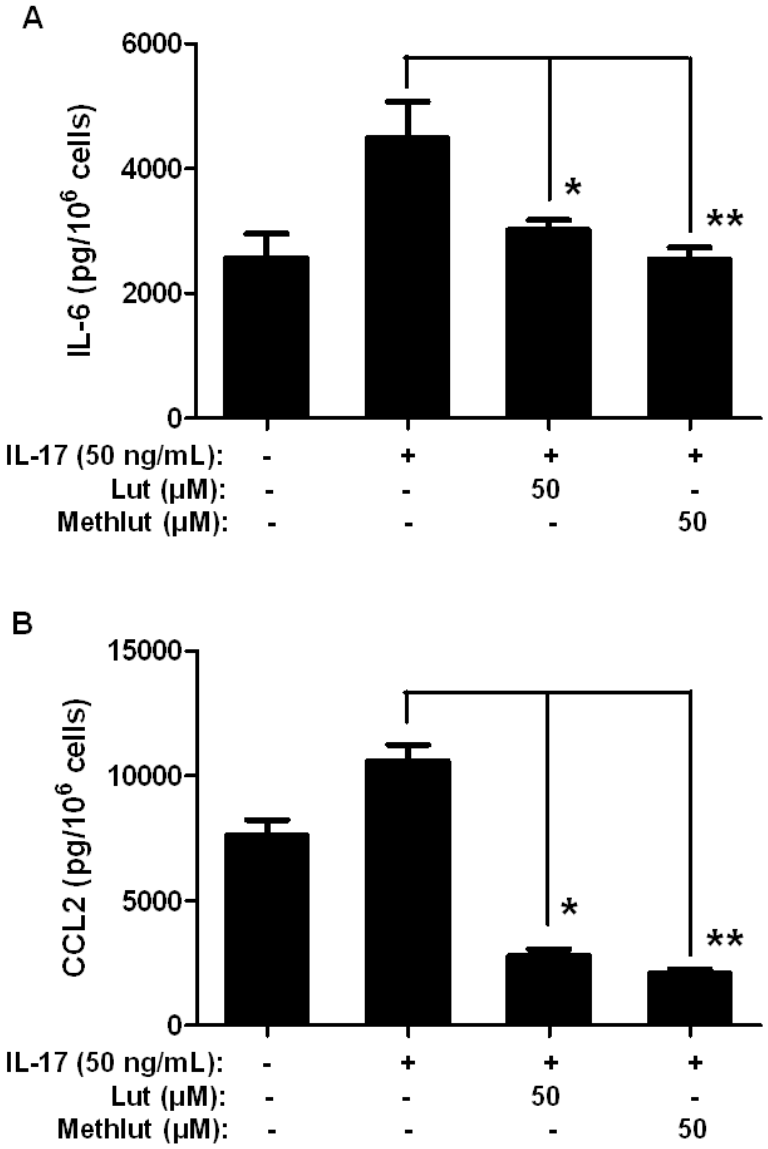


Luteolin and methoxyluteolin inhibit IL-17-triggered IL-6 and CCL2 release from HaCaT cells.

Apart from TNF, keratinocytes can also be triggered by other stimuli, such as the cytokine IL-17, which also participates in Ps pathogenesis[121]. We examined the effect of luteolin and methoxyluteolin on mediator release triggered by IL-17. Stimulation of HaCaT cells with IL-17 (50 ng/mL, 24 h) induces significant IL-6 and CCL2 production (Fig. 3.5). Preincubation with luteolin and methoxyluteolin (50 μM, 30 min) blocks IL-17-triggered IL-6 release (Fig. 3.5 A). The two flavones also decrease CCL2 release below the basal level (Fig. 3.5 B).

Figure 3.5. Effect of luteolin and methoxyluteolin on IL-17-triggered IL-6 and CCL2 release from HaCaT cells

HaCaT cells were preincubated with luteolin or methoxyluteolin (50 μM) for 30 min before stimulation with IL-17. The release of (A) IL-6 and (B) CCL2 was measured by ELISA at 24 h after IL-17 stimulation (50 ng/mL). * $p<0.05$, ** $p<0.01$. Lut, luteolin; Methlut, methoxyluteolin.

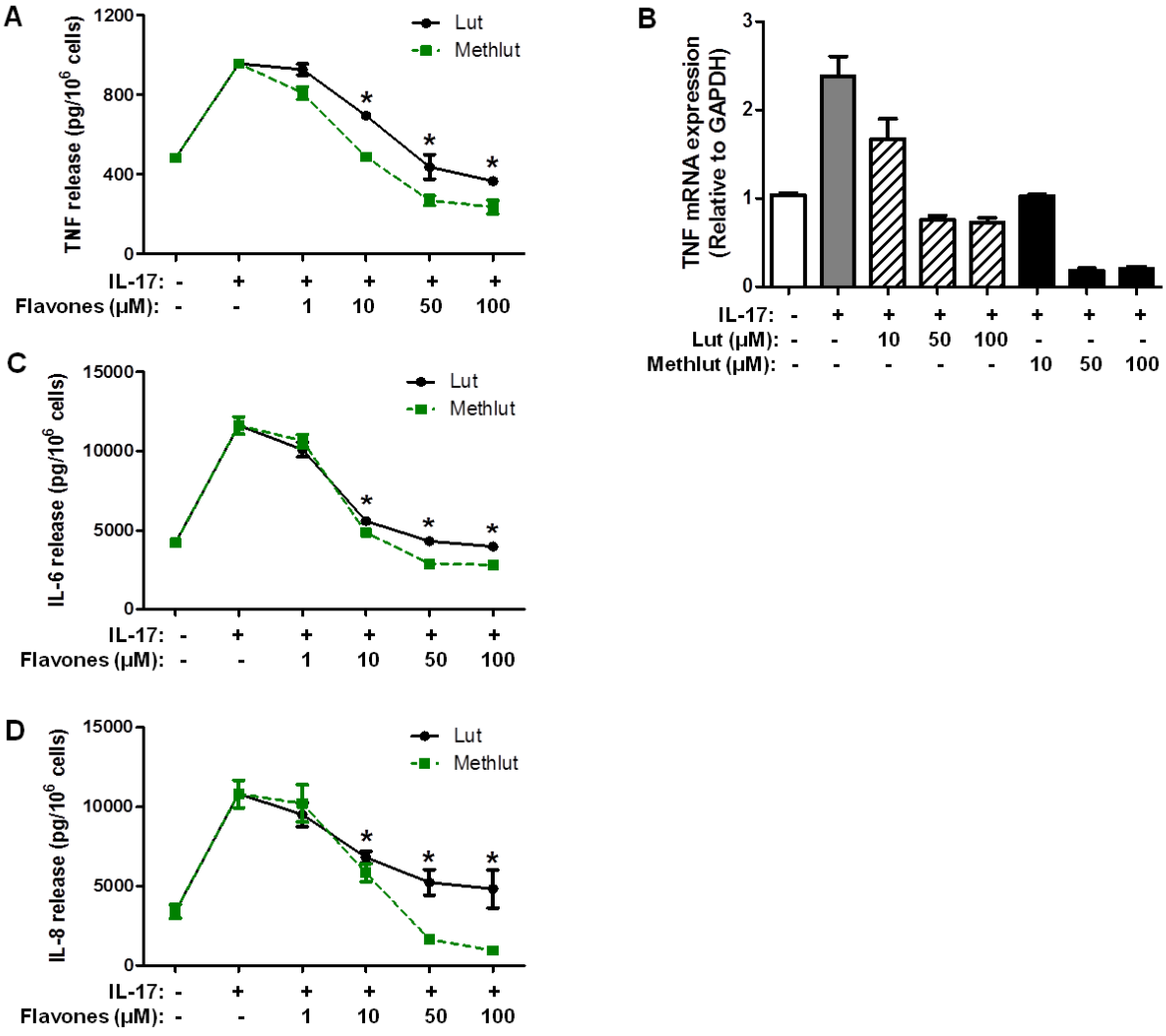


Luteolin and methoxyluteolin inhibit IL-17-triggered TNF, IL-6 and IL-8 release from NHEKs

We also examined the inhibitory effect of the two flavones on mediator release triggered by IL-17 in primary keratinocytes. Stimulation of NHEKs with IL-17 (50 ng/mL, 24 h) induces TNF, IL-6 and IL-8 release (Fig. 3.6 A, C, D). Preincubation with luteolin and methoxyluteolin (1-100 μM, 30 min) concentration-dependently inhibit IL-17-triggered mediator release. Methoxyluteolin is more potent than luteolin, especially in blocking IL-8 release (Fig. 3.6 D). The two flavones also decrease IL-17-triggered TNF mRNA expression in NHEKs (Fig. 3.6 B, n=2). Again, NHEKs are more sensitive to the inhibitory actions of the two flavones than HaCaT cells.

Figure 3.6. Effect of luteolin and methoxyluteolin on IL-17-triggered TNF, IL-6 and IL-8 release from NHEKs

NHEKs were preincubated with luteolin (solid lines) or methoxyluteolin (dashed lines) (1-100 μM, 30 min) before stimulation with IL-17 (50 ng/mL). The release of (A) TNF, (C) IL-6 and (D) IL-8 was measured by ELISA at 24 h after IL-17 stimulation. In (B), the mRNA expression of TNF was measured by qRT-PCR at 6 h after IL-17 stimulation. * $p<0.05$. Lut, luteolin; Methlut, methoxyluteolin.

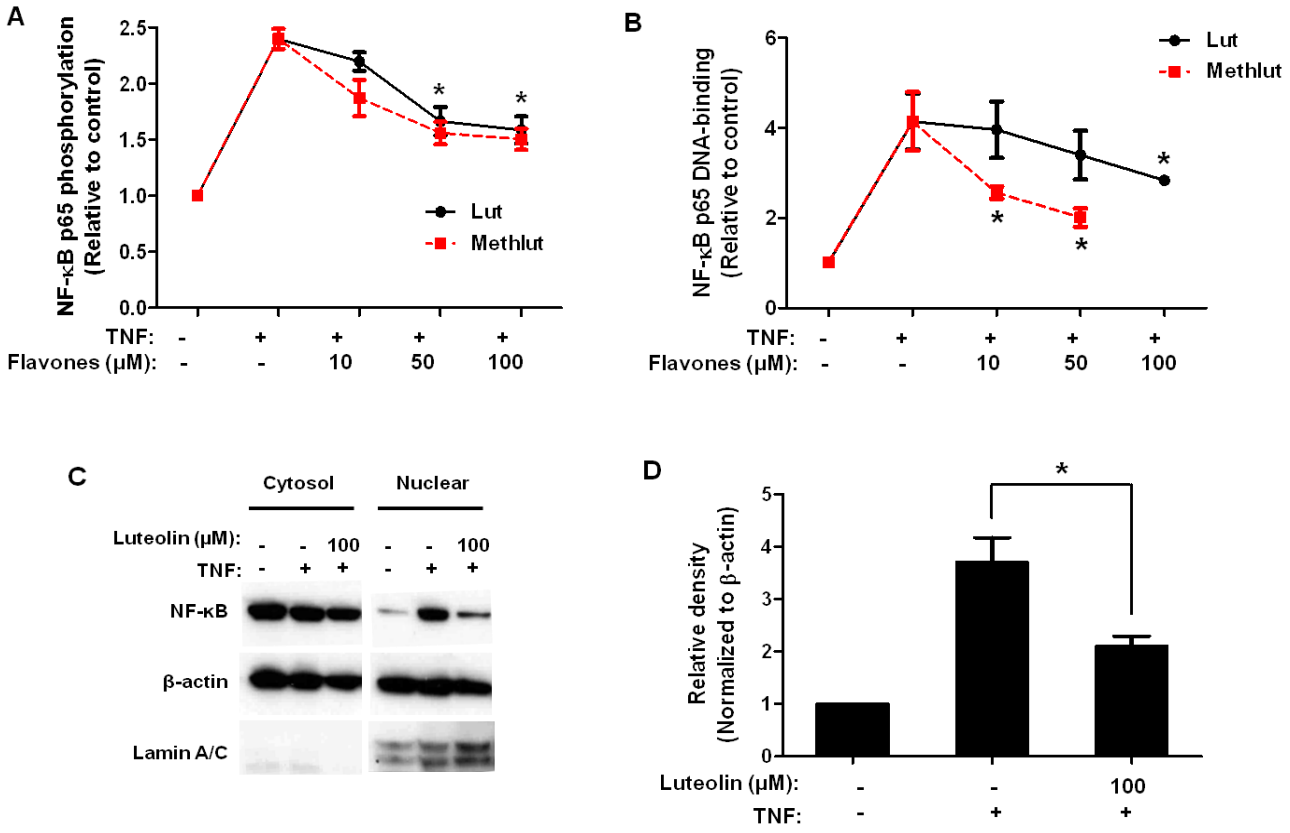


Luteolin and methoxyluteolin inhibit TNF-triggered NF-κB activation

Stimulation of HaCaT cells with TNF (50 ng/mL, 15 min) rapidly causes NF-κB p65 phosphorylation, which is significantly reduced with luteolin and methoxyluteolin preincubation (50 and 100 μM, 6 h) by 50% compared to control cells (Fig. 3.7 A). TNF stimulation (50 ng/mL, 15 min) also increases NF-κB p65 DNA-binding activity in the nucleus, which is significantly decreased by luteolin and methoxyluteolin preincubation (50 and 100 μM, 6 h) (Fig. 3.7 B). This is further supported by Western blot analysis showing significant reduction of NF-κB p65 nuclear translocation in luteolin (100 μM)-pretreated cells (Fig. 3.7 C, D). To verify that there was no cross-contamination of nuclear and cytosolic extracts, a nuclear protein lamin A/C, was probed and shown to be present in the nuclear extract only, and absent in the cytosolic extract (Fig. 3.7 C).

Figure 3.7. Effect of luteolin and methoxyluteolin on TNF-triggered NF-κB activation in HaCaT cells

HaCaT cells were preincubated with either luteolin or methoxyluteolin (10-100 μM, 6 h) before stimulation with TNF (50 ng/mL, 15 min). (A) Whole cell lysates were collected and phosphorylation levels of NF-κB p65 were determined using a Multi-Target Sandwich ELISA kit and are presented as fold change relative to control. In another set of experiments following the same treatments, cytosolic and nuclear fractions were separated, and (B) NF-κB p65 DNA-binding activities in the nuclear fractions were measured and expressed as fold change relative to control. (C) Nuclear translocation of NF-κB p65 was determined using Western blot and quantified by densitometry (D). The Western blot shown is representative of 3 independent experiments. * $p<0.05$. Lut, luteolin; Methlut, methoxyluteolin.



Luteolin reduces TNF-triggered mRNA expression of NFKB1 and RELA

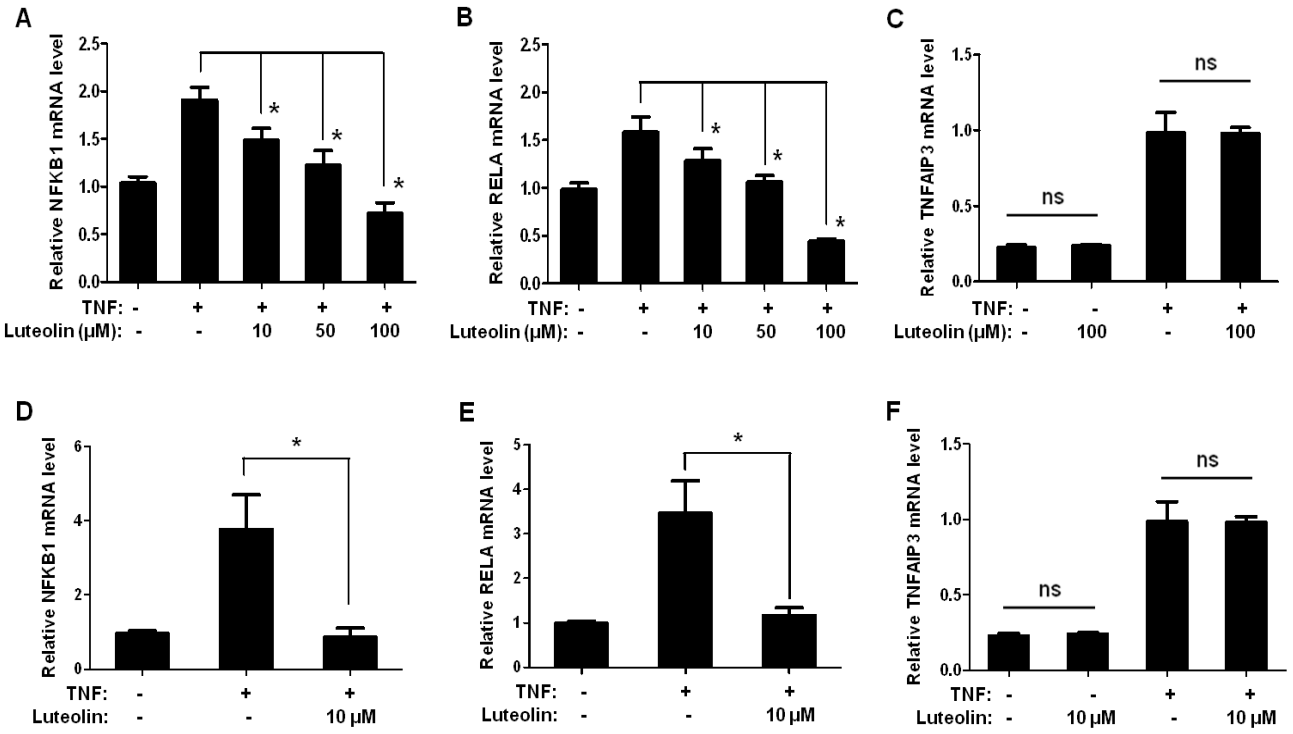
We also studied for the first time the effect of luteolin on mRNA expression of three genes involved in TNF-triggered NF-κB activation, NFKB1 (encoding NF-κB p50 subunit), RELA (encoding v-rel reticuloendotheliosis viral oncogene homolog A or NF-κB p65 subunit), and TNFAIP3 (encoding tumor necrosis factor alpha-induced protein 3).

TNF significantly induces NFKB1 and RELA mRNA expression in HaCaT cells, which is reduced by preincubation with luteolin (10-100 μM, 30 min, Fig. 3.8 A, B) in a concentration-dependent manner. Moreover, TNF stimulation dramatically increases the mRNA expression of TNFAIP3, which is critical for blocking TNF-stimulated NF-κB responses and reducing inflammation in a feedback loop[227]. Treatment with luteolin alone, or preincubation before TNF stimulation, has no effect on TNFAIP3 mRNA expression in HaCaT cells (Fig. 3.8 C).

In primary NHEKs, TNF triggers more than 3-fold increase of NFKB1 and RELA mRNA levels compared to control cells (Fig. 3.8 D, E), which is a more prominent increase than in HaCaT cells. Luteolin preincubation (10 μM, 30 min) effectively decreases mRNA expression of NFKB1 and RELA back to the basal level. TNF induces a very similar increase of TNFAIP3 mRNA expression in NHEKs as compared to HaCaT cells, where luteolin again has no inhibitory effect (Fig. 3.8 F).

Figure 3.8. Effect of luteolin on TNF-stimulated mRNA expression of NFKB1 (NF-κB p50) and RELA (NF-κB p65) in human keratinocytes

The effect of luteolin on mRNA expression levels of genes involved in TNF-stimulated NF-κB activation was investigated using qRT-PCR. HaCaT cells were preincubated with luteolin (10-100 μM, 30 min) before stimulation with TNF (50 ng/mL, 6 h): (A) NFKB1, (B) RELA, (C) TNFAIP3. NHEKs were preincubated with luteolin (10 μM, 30 min) before stimulation with TNF (50 ng/mL, 6 h): (D) NFKB1, (E) RELA, (F) TNFAIP3. * $p<0.05$; ns = not statistically significant.

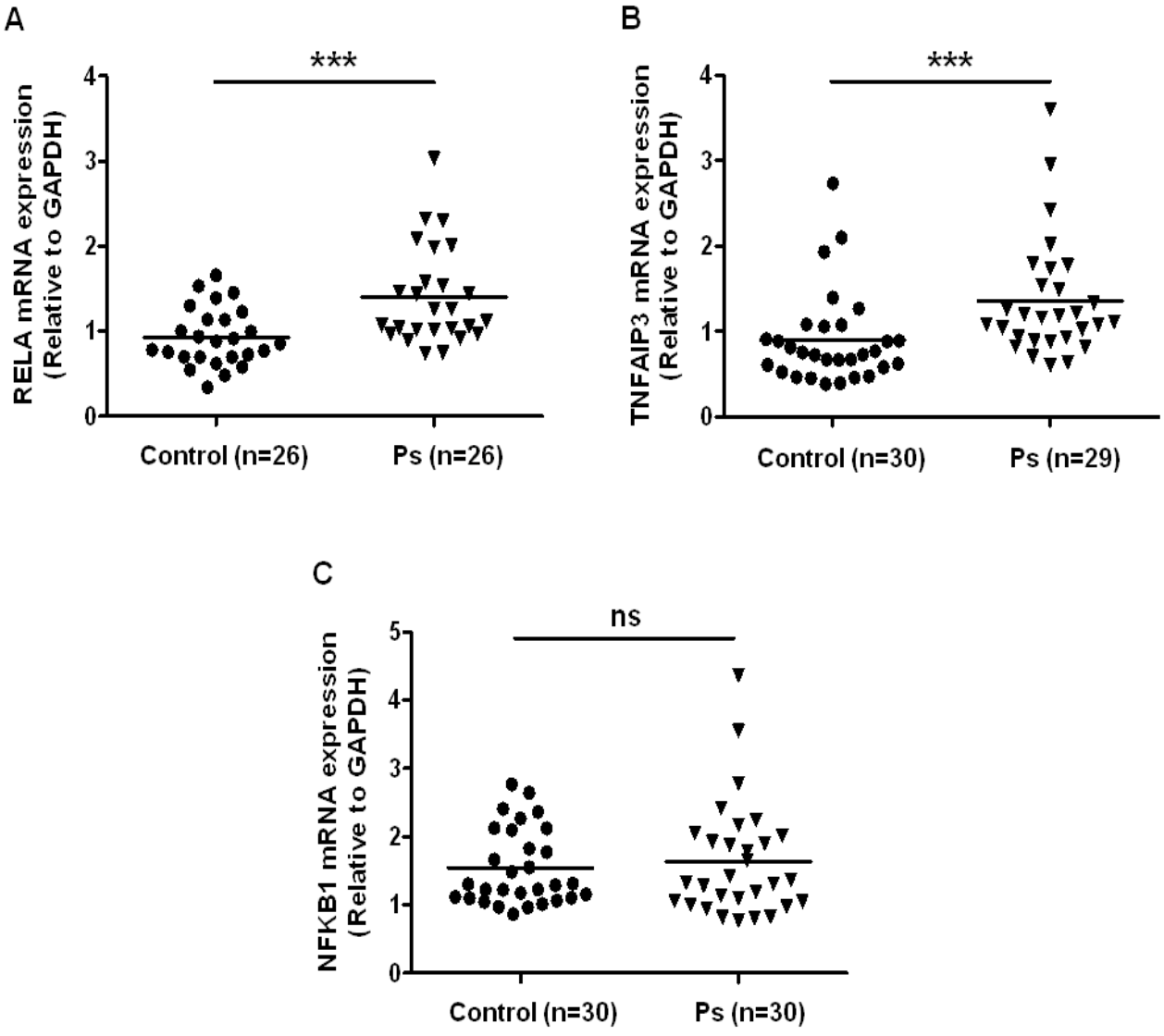


Gene expression of RELA and TNFAIP3 is increased in Ps skin

We further investigated the mRNA expression of NFKB1, RELA and TNFAIP3 in skin biopsies obtained from Ps patients (n=26-30). There was no statistical difference in the mean age between Ps patients and healthy controls. In Ps lesional skin, RELA ($p=0.0007$, Fig. 3.9 A) and TNFAIP3 mRNA expression ($p=0.0004$, Fig. 3.9 B) was increased by 1.5-fold as compared to controls. In contrast, there is no statistically significant difference in NFKB1 mRNA expression between Ps patients and controls (Fig. 3.9 C).

Figure 3.9. Gene expression of RELA and TNFAIP3 is increased in Ps skin

Total RNA was extracted from Ps lesional skin biopsies and qRT-PCR was performed for mRNA expression levels of (A) RELA, (B) TNFAIP3, and (C) NFKB1, which were normalized to human GAPDH as an endogenous control. *** $p<0.001$; ns = not statistically significant.



Luteolin and methoxyluteolin reduce keratinocyte proliferation without affecting ATP production

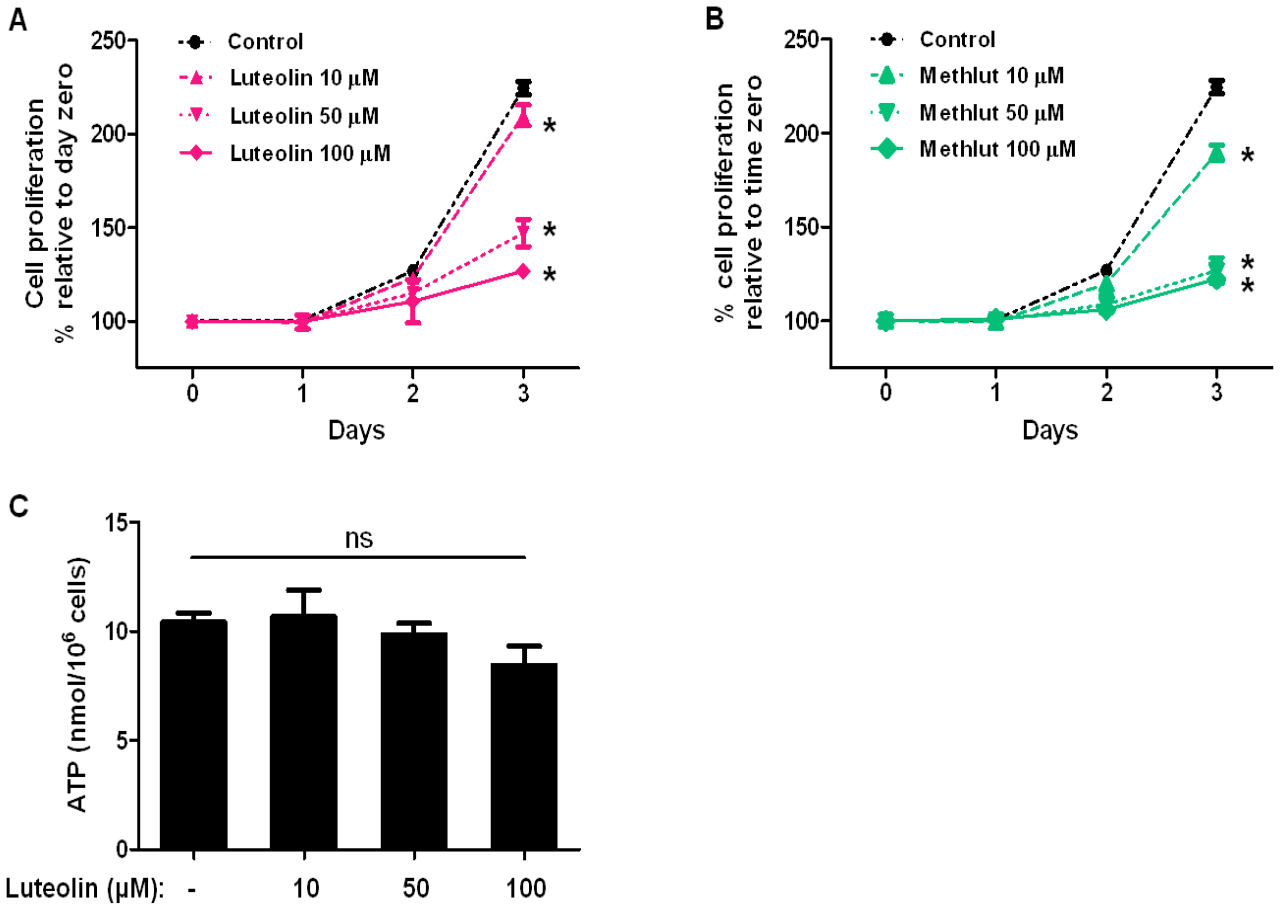
We examined the effect of luteolin and methoxyluteolin on keratinocyte proliferation using the XTT-based assay. The proportion of XTT reduction is directly proportional to the number of viable cells in culture. Preincubation with luteolin or methoxyluteolin (10 μM) significantly reduces keratinocyte proliferation compared to control cells (treated with 0.1% DMSO) after 3-day incubation (Fig. 3.10 A, B). The inhibition is stronger with 50 μM and 100 μM of the two flavones. It is important to note that cell viability remains above 90% (data not shown). Interestingly, luteolin has no effect on proliferation of NHEKs (data not shown). Noteworthy, cytokine gene and protein expression are measured at 6 and 24 h, respectively, when there is no effect on cell proliferation by luteolin or methoxyluteolin.

In order to investigate if luteolin has any damaging effect on cellular metabolic activity, we also measured intracellular ATP levels. In HaCaT cells, although luteolin incubation (100 μM, 72 h) reduces intracellular ATP level by 13% (Fig. 3.10 C) compared to control cells, however, this effect is not statistically significant.

The two flavones do not affect proliferation and intracellular ATP levels of normal NHEKs (data not shown).

Figure 3.10. Effect of luteolin and methoxyluteolin on HaCaT keratinocyte proliferation and ATP production

HaCaT cells were incubated with luteolin or methoxyluteolin (10-100 µM) for up to 3 days. Cell proliferation was examined using a XTT-based assay. (A) Luteolin. (B) Methoxyluteolin. (C) Intracellular ATP content was determined using an ATP assay kit. * $p<0.05$; ns = not statistically significant. Methlut, methoxyluteolin.



Discussion

Ps is a debilitating disease involving increased inflammatory cytokine production and keratinocyte hyperproliferation[80, 228]. Despite success in Ps treatment with anti-proliferative and anti-TNF therapies, many patients experience serious side effects and drug resistance[80, 229]. New biological therapies have revolutionized the treatment for Ps, but none of these treatments address release of cytokines or keratinocyte proliferation. There is also a need for less invasive approaches, especially for patients with mild Ps symptoms. Here we report that the two flavones, luteolin and methoxyluteolin, significantly inhibit both TNF- and IL-17-triggered production of inflammatory mediators (TNF, IL-6, IL-8, CCL2 and VEGF) from human keratinocytes and decrease activation of the transcription factor NF-κB. We also report for the first time to our knowledge that luteolin decreases mRNA expression of two genes (NFKB1 and RELA) encoding two subunits (p50 and p65, respectively) in the NF-κB protein complex, that is important for NF-κB activation.

The mediators TNF, IL-17, IL-6, IL-8, CCL2 and VEGF play a pivotal role in the initiation and progression of chronic inflammation[80]. Our finding is significant that luteolin and methoxyluteolin exert inhibitory effects at the transcriptional level, which were not apparently known to date. Previously, it has been reported that the flavonol quercetin has the ability to actively accumulate in the nucleus and modify the activity of numerous transcription factors[211]. In our studies, luteolin readily enters the cells (data presented in Chapter 4). As structurally-related flavones to quercetin, we suggest that

luteolin and methoxyluteolin might have similar actions and regulate gene transcription inside the nucleus.

We also show that luteolin inhibits TNF-triggered activation of the transcription factor NF-κB at both the protein and transcriptional levels. NF-κB is an inducible transcription factor constitutively expressed in HaCaT cells[230]. TNF induces NF-κB activation, which sets up an autocrine signaling loop that leads to further TNF secretion and sustained NF-κB activation[231]. Previous studies also suggest that the NF-κB pathway plays an essential role in Ps progression[232, 233], where there is marked elevation of active phosphorylated NF-κB p65 in Ps lesional skin[106]. Moreover, polymorphisms in two genes downstream of TNF that regulate NF-κB activation (TNFAIP3, TNIP1) are associated with increased susceptibility to Ps[234]. In addition, skin biopsies from Ps patients treated with the anti-TNF therapy etanercept for 1, 3 and 6 months showed a significant reduction of phosphorylated NF-κB p65 [106] implying that blockade of TNF activity leads to NF-κB downregulation.

Our results also show that mRNA expression of RELA (NF-κB p65 subunit) and TNFAIP3 is significantly increased in Ps lesional skin. These two factors are necessary for NF-κB-mediated induction of inflammatory mediator expression[234]. Despite being a modest effect, long-standing Ps could downregulate these NF-κB-related genes. In addition, the 15-day wash-out period before taking skin biopsies from Ps patients might not be long enough for NF-κB transcriptional activity to fully recover.

Ps is also characterized by increased number of lesional skin mast cells[235], the only cell type that rapidly secretes preformed TNF [208, 236], which could stimulate keratinocytes. Luteolin has been shown to inhibit mast cell degranulation [153] and cytokine release[237]. Luteolin also inhibits mast cell-dependent stimulation of activated T cells[238, 239], a key feature in Ps pathogenesis[80]. Moreover, the marked inhibition of IL-6 gene expression and secretion by luteolin and methoxyluteolin is particularly important since IL-6 is required to drive maturation of Th17 cells[240], which are also involved in Ps pathogenesis[228]. Luteolin could antagonize the activation of and interactions between multiple cell types involved in Ps, including keratinocytes, MC and T cells. In addition, increased keratinocyte proliferation is a characteristic of Ps dermis. Here we demonstrate that luteolin and methoxyluteolin significantly decrease HaCaT, but not normal keratinocyte proliferation without causing apparent cell death.

The flavones luteolin and methoxyluteolin are lipid-soluble and could be developed into topical formulations that easily penetrate the skin. Luteolin is generally safe[241-243], and can even protect against chemically-induced hepatotoxicity [177] and nephrotoxicity[178]. Given that flavonoids also have anti-cancer properties[244, 245], luteolin or methoxyluteolin could be used together with anti-TNF agents both for synergism and for protection against the cancer-inducing side effects of such agents.

In conclusion, the present findings suggest for the first time, to our knowledge, that both luteolin and methoxyluteolin inhibits the production of inflammatory mediators IL-6, IL-8 and VEGF from TNF-triggered keratinocytes and decreases NF-κB activation

at both the transcriptional and translational levels. These two flavones also decrease keratinocyte proliferation without affecting intracellular energy production. Hence, luteolin and methoxyluteolin have the potential to be developed into safer and more effective alternative therapies for the treatment of inflammatory skin diseases, including Ps.

Chapter 4. Cellular Uptake and Distribution of Luteolin

Introduction

As shown in the above results, luteolin at the concentration tested (10-100 μM) effectively blocks activation of both human mast cells and keratinocytes. We further studied the cellular uptake of luteolin in order to determine how much of the administered luteolin actually enters the cells, as well as the distribution into different cellular compartments (such as the mitochondria and nuclei). Previously, epigallocatechin gallate (EGCG) has been shown to accumulate in the interior of mitochondria in rat neuronal cells[246]. In addition, quercetin was rapidly absorbed by Jurkat T cells and their isolated mitochondria, as well as by the mitochondria of quercetin-pretreated cells[247]. Moreover, quercetin was actively absorbed into the nuclei of epithelial cells[211]. It is likely that luteolin, as a structurally-related flavone to quercetin with one less hydroxyl group, will be associated with mitochondria or nuclei.

Methods

Extraction of luteolin from LAD2 mast cells

We chose an incubation time of 30 min because it is the shortest preincubation time used for assessing effects of luteolin on mast cell and keratinocyte activation (see Chapters 2 and 3). After incubation with luteolin (10 μM and 50 μM) for 30 min, LAD2 mast cells (1×10^7 cells) were washed twice with ice-cold PBS, resuspended in PBS containing 0.5% Triton X-100 and lysed with a sonicator. Luteolin from whole cell lysates was extracted with ethyl acetate, and taken to dryness by vacuum evaporation protected from light, then dissolved in 5 μL of DMSO and diluted with 195 μL of acetonitrile (30% in distilled water, with 100 μM narigenin as internal standard). The resulting solutions were centrifuged at full speed for 10 min at 4°C. Supernatants were transferred to glass vials. Luteolin content in the supernatants was assayed by high pressure liquid chromatography (HPLC) analysis.

To determine mitochondrial and nuclear uptake of luteolin, mitochondria or nuclei were isolated immediately from luteolin-loaded cells after 30-min incubation. The same extraction steps were then performed. Luteolin contents in the isolated mitochondrial or nuclear fractions were also assayed by HPLC analysis.

HPLC analysis

HPLC analysis was conducted using an Agilent 1100 series HPLC system (Agilent, Palo Alto, CA). The column was a 4-μm, C-18, 250×4.6 mm Synergi Hydro-RP 80A column (Phenominex, Torrance, CA). Separation was achieved using a gradient comprised of 0.1% formic acid and acetonitrile. The running gradient was started with 20% acetonitrile for 2 min, increasing to 60% at 15 min, then returned to 20% acetonitrile at 16 min. The column was re-equilibrated with 20% acetonitrile for 9 min prior to the next injection. The flow rate was held constant at 1.0 mL/min throughout. Column effluent was monitored with an Agilent 1100 series UV detector set at 370 nm (Agilent). All solvents were HPLC grade (Sigma-Aldrich), and water was purified via a Milex Q-plus system (Millipore).

An 8-point standard curve was constructed with a final luteolin concentration range of 0.05 – 50 μM. Standards were prepared in blank cell lysates and extracted by the same procedures described above. Luteolin concentrations in the extracted solutions were determined from the standard curve. Actual amounts of luteolin taken up by cells and mitochondria were calculated and expressed as nmol/10^7 cells. Hypothetical intracellular and mitochondrial concentrations of luteolin were also calculated using published values for cellular [248] and mitochondrial [249] volume, respectively, assuming that the entire fraction of the cell-associated luteolin was unbound. It is understood that other cellular components may also concentrate the flavonoids in which case calculations of any concentration could be misleading.

Mass spectrometry analysis for assaying purity of methoxyluteolin

Luteolin was obtained from Sigma Aldrich, and is >97% pure. Methoxyluteolin used here was obtained from Pharmascience Nutrients (Clear Water, FL). An electron-spray mass spectrometry analysis was conducted to examine if the methoxyluteolin powder is pure.

Results

Luteolin readily enters LAD2 mast cells and is associated with mitochondria

Fig. 4.1 is a representative standard curve obtained from the HPLC analysis, from where luteolin content in the cell lysates was calculated. After 30 min incubation with luteolin (10 μM), the concentrations of luteolin detected in whole cell extracts and mitochondrial extracts are around 20 μM and 195 μM, respectively (Table 4.1 A, n=1). The estimated amount of luteolin taken up into the cells was 2.08 nmol/10^7 cells, which is about 1% of the total amount of luteolin administered (200 nmol, Table 4.1 A). Luteolin is also detected in the mitochondrial fraction with an approximate amount of 0.52 nmol/10^7 cells.

After 30 min incubation with 50 μM luteolin, intracellular concentration of luteolin reaches around 900 μM, and luteolin concentration in the mitochondrial fraction is above 1 mM (Table 4.1 B, n=3), which are much higher compared to the concentrations obtained from incubation with 10 μM luteolin. The estimated amount of luteolin taken up into the cells was 90.58 nmol/10^7 cells, which is about 10% of the total amount of luteolin administered (1000 nmol, Table 4.1 B). Luteolin is again detected in the mitochondrial extract at about 3.46 nmol/10^7 cells. More interestingly, luteolin is also detected in the nuclear extract at about 1.31 nmol/10^7 cells (n=2, Table 4.1 B).

To further verify mitochondrial uptake, intact mitochondria were first isolated from untreated cells, and then exposed to the same luteolin incubation employed with whole cells (50 μM, 30 min, n=1, Table 4.1 B). Luteolin is detected in the isolated

mitochondrial extract at about 2 nmol/10^7 cells, which is comparable to that obtained from mitochondrial extract of whole cells (3.46 nmol/10^7 cells, Table 4.1 B).

Figure 4.1. Representative standard curve of luteolin from the HPLC analysis

HPLC analysis was conducted using an Agilent 1100 series HPLC system (Agilent, Palo Alto, CA). The column was a 4-µm, C-18, 250×4.6 mm Synergi Hydro-RP 80A column (Phenominex, Torrance, CA). Separation was achieved using a gradient comprised of 0.1% formic acid and acetonitrile. The running gradient started with 20% acetonitrile for 2 min, increasing to 60% at 15 min, then returned to 20% acetonitrile at 16 min. The column was re-equilibrated with 20% acetonitrile for 9 min prior to the next injection. The flow rate was held constant at 1.0 mL/min throughout. Column effluent was monitored with an Agilent 1100 series UV detector set at 370 nm (Agilent). All solvents were HPLC grade (Sigma-Aldrich). Luteolin standards (0.1 – 10 µM) were prepared in blank LAD2 cell lysates.

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
0.10	1	0.10	97.1	N/A	N/A
0.25	1	0.28	112.5	N/A	N/A
0.50	1	0.46	91.8	N/A	N/A
1.00	1	0.95	95.1	N/A	N/A
5.00	1	4.69	93.9	N/A	N/A
10.00	1	10.95	109.5	N/A	N/A

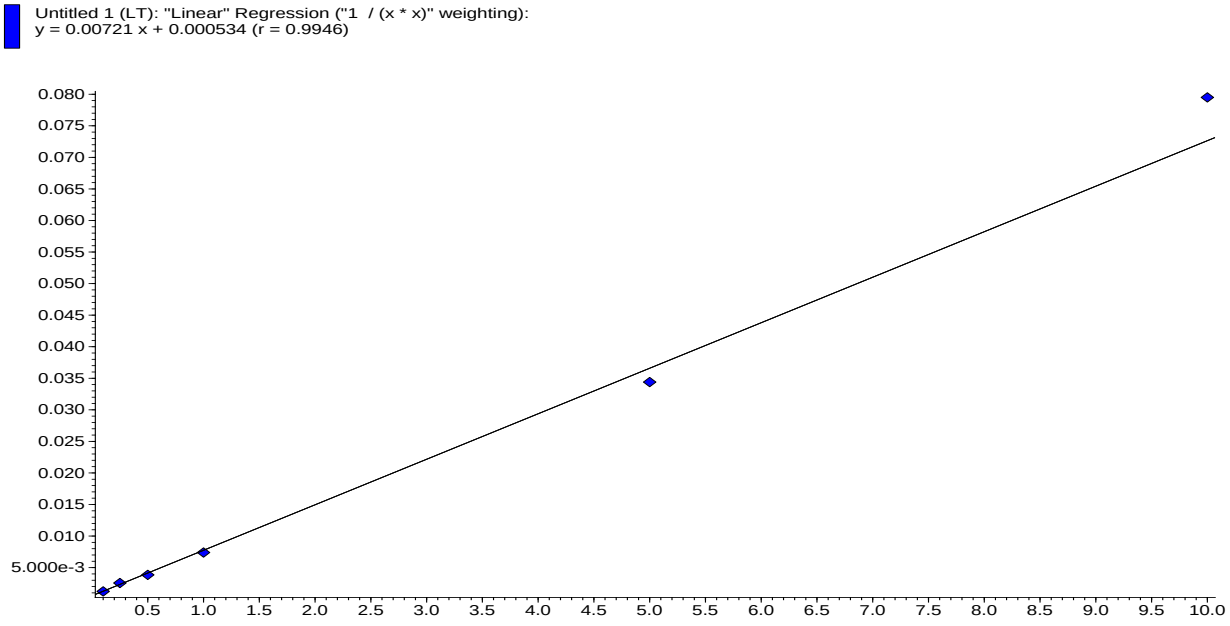


Table 4.1. Luteolin is associated with mitochondria of LAD2 mast cells

After 30 min incubation with 10 μM (A) or 50 μM (B) luteolin, LAD2 cells were washed twice with ice-cold PBS and resuspended in PBS containing 0.5% Triton-X 100 and lysed with a sonicator. Luteolin was extracted and luteolin content in whole cell lysates were measured by HPLC analysis. The same procedures were performed with isolated mitochondrial or nuclear extracts after whole cell luteolin exposure. In another set of experiments, mitochondria or nuclei were first isolated from untreated LAD2 cells, then incubated with luteolin (50 μM) for 30 min. Approximate luteolin concentrations were determined from a standard curve constructed with luteolin standard dissolved in DMSO. Final amount of luteolin detected in cellular extracts are expressed as "nmol per 10^7 cells".

A

Luteolin 10 μM	No. of experiments (n)	Total amount (nmol) administered in 20 mL media	Calculated concentration from standard curve (μM)	Cellular concentration (μM)	Amount in cells (nmol/10^7 cells)	Mean	SD
Whole cell extract	1	200	1.038	20.76	2.08	2.08	
Mitochondrial extract	1	200	0.261	195.14	0.52	0.52	

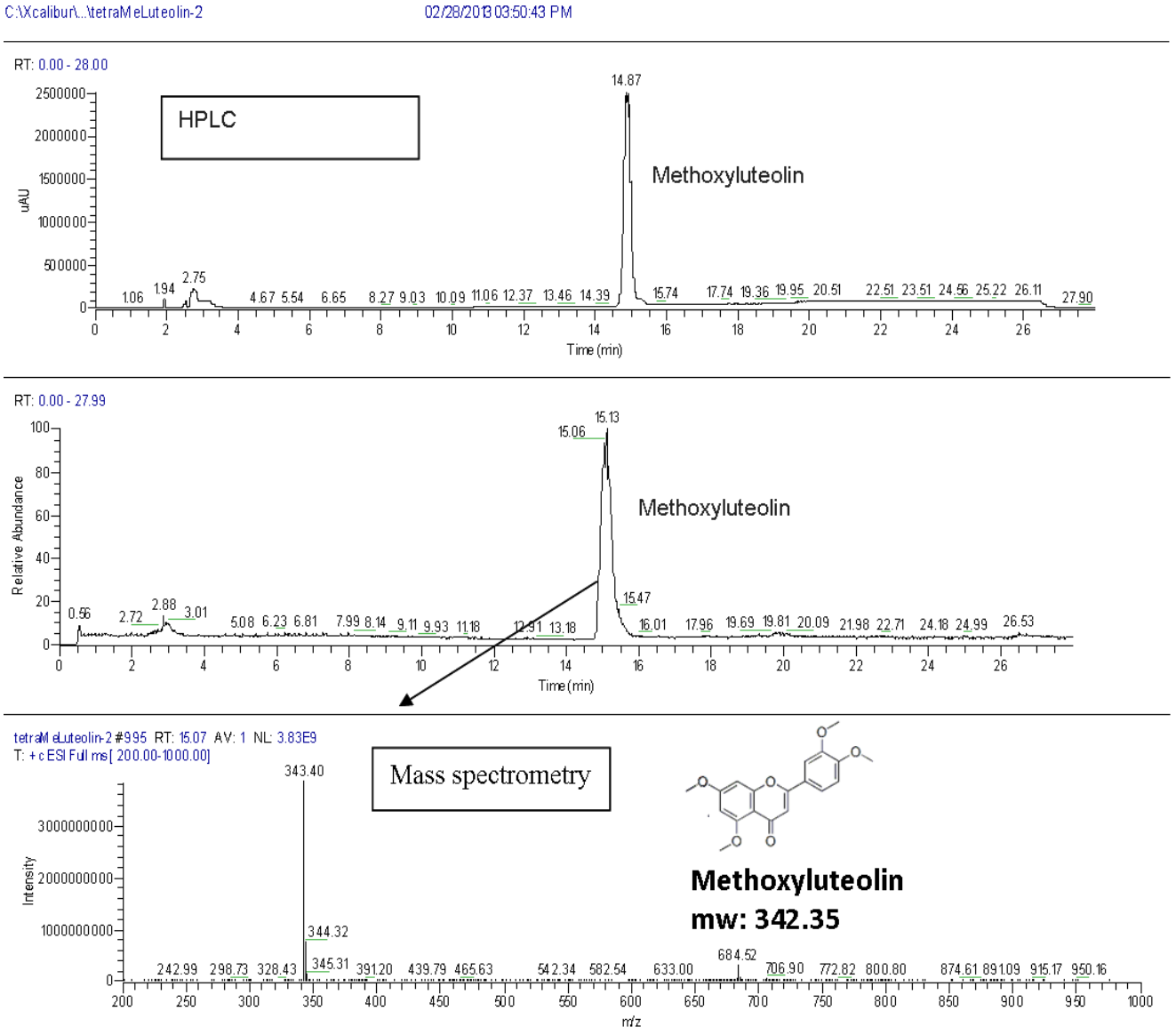
B

Luteolin 50 μM	No. of experiments (n)	Total amount (nmol) administered in 20 mL media	Calculated concentration from standard curve (μM)	Cellular concentration (μM)	Total amount in cells (nmol/10^7 cells)	Mean	SD
Whole cell extract	3	1000	31.700	634.00	63.40	90.58	25.89
		1000	46.700	934.00	93.40		
		1000	57.470	1149.40	114.94		
Mitochondrial extract	3	1000	2.020	1510.28	4.04	3.46	0.51
		1000	1.630	1218.69	3.26		
		1000	1.534	1146.92	3.07		
Nuclear extract	2	1000	0.663		1.33	1.31	
		1000	0.648		1.30		
Isolated mitochondria	1	1000	1.001		2.00	2.00	
Isolated nuclei	1	1000	NF		NF	NF	

Methoxyluteolin is pure

Mass spectrometry analysis shows that methoxyluteolin is pure (Fig. 4.2).

Figure 4.2. Representative spectrum showing the mass analysis of 3',4',5,7-tetramethoxyluteolin by LC/MS.



Discussion

In our experiments, we incubated 10^7 LAD2 cells with two concentrations of luteolin, 10 μM and 50 μM. We show that luteolin is readily detected in whole cell lysates, as well as mitochondrial and nuclear extracts in human LAD2 mast cells within 30 min incubation. In a previous study using human T cells, quercetin was detected in cellular extracts within 20 seconds, and reached a plateau of maximum detected amount at 30 seconds[247], suggesting that flavonoids are generally rapidly taken up by human cells *in vitro*.

Here, the final amounts of luteolin detected in the whole cell extracts are calculated to be about 0.2 and 9 nmol per 10^6 cells after incubation with luteolin at 10 μM and 50 μM, respectively. In comparison to the previous study, cellular amounts of quercetin reach about 0.5 and 3 nmol per 10^6 cells after incubation with quercetin for 10 min at 10 μM and 50 μM, respectively[247]. Thus, at 10 μM, the cellular uptake of quercetin and luteolin are comparable, but at a higher concentration (50 μM) luteolin penetrates cells more efficiently than quercetin. This might account for the finding that luteolin is more effective than quercetin in inhibiting mast cell activation (Fig. 2.3).

According to our studies, only 10% of administered luteolin enters the cells after 30 min incubation. This result leads to two important conclusions. Firstly, luteolin can rapidly penetrate cells with sufficient amount to effectively block mast cell degranulation. On the other hand, it is also possible that luteolin is embedded in the lipid bilayers of the plasma membrane due to its hydrophobic character. If this is the case, then luteolin may

exert its inhibitory effect by interacting with surface receptors or signaling molecules residing in the plasma membrane. Secondly, the findings indicate that a large amount of luteolin is retained outside of the cells in the culture media, implying that luteolin avidly binds to hydrophobic proteins such as human serum albumin (which is present in culture media), potentially through multiple phenolic groups allowing hydrogen bonds and hydrophobic interactions. This is physiologically relevant since serum albumin is considered a "taxi" in the systemic circulation for binding and transporting molecules with lower water solubility[250], and later on release those molecules where needed. Hence, with the help of albumin, luteolin could reach distant tissues such as the skin.

In the previous quercetin study, the authors also show that cellular uptake of quercetin is linear[247]. In our results, we only tested luteolin uptake at two specific concentrations. Incubation with more concentrations of luteolin or methoxyluteolin would reveal whether cellular uptake is also linear for these two flavones.

More interestingly, here we show that luteolin is detected in both mitochondrial and nuclear extracts. Another group had previously shown that quercetin accumulates into nuclei of HepG2 hepatocytes, especially at nucleolar areas, and does not colocalize with either actin or condensed mitotic chromatin[211]. Our results from the HPLC experiments are not sufficient to conclude that luteolin truly enters the mitochondria and nuclei. Considering the high lipid solubility of luteolin, a large fraction of the flavone is very likely imbedded in lipid membranes or bound to other intracellular molecules.

Nevertheless, the results clearly suggest that luteolin readily penetrates the plasma membrane and is “associated” with mitochondria and nuclei once inside the cells.

To further validate the cellular uptake of luteolin, and based on the fact that flavonoids are polyphenolic compounds with intrinsic fluorescence[251], fluorescence or confocal microscopy could be used to monitor the real-time uptake and distribution of luteolin into different cellular compartments in live cells.

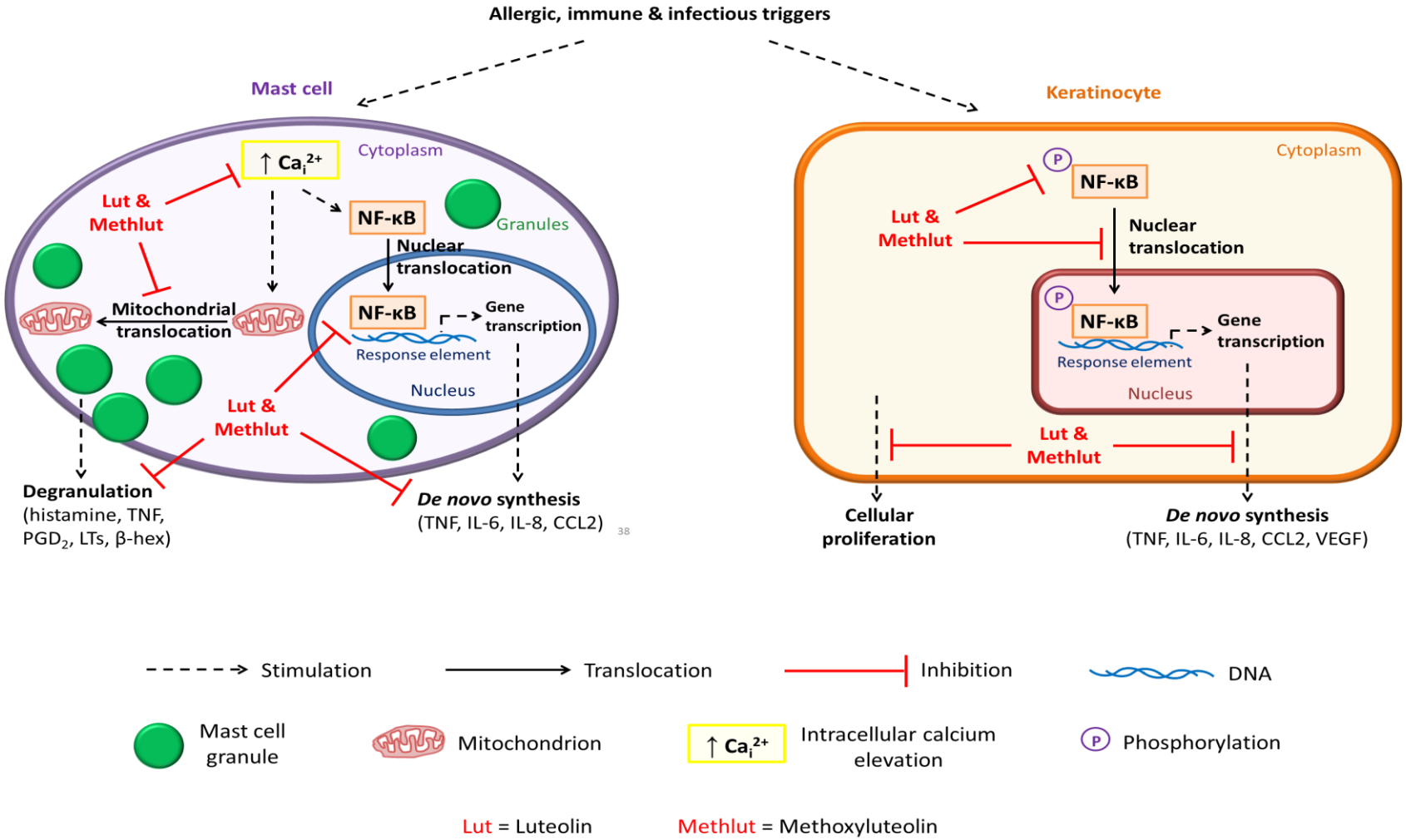
In addition, similar HPLC assay procedures are being developed for detecting methoxyluteolin in cellular extracts to compare the rate and extent of uptake of methoxyluteolin to that of luteolin. One of the reasons why methoxyluteolin is more effective than luteolin in inhibiting mast cells and keratinocytes could be due to a better cellular uptake of methoxyluteolin due to its increased hydrophobic character, as compared to that of quercetin and luteolin.

Chapter 5. Conclusions and Clinical Significance

Overall Conclusions and Significance

Here we identified two natural flavones, luteolin and its structural analogue 3',4',5,7-tetramethoxyluteolin (methoxyluteolin), as potent inhibitors of mast cell immediate degranulation, as well as delayed synthesis and release of IL-6, IL-8, CCL2, TNF and VEGF from human mast cells and keratinocytes triggered by different stimuli. As illustrated in Fig. 5.1, the two flavones potently block intracellular calcium elevation in mast cells, which is essential for their degranulation and inflammatory mediator release. We also show for the first time that luteolin reduces SP-triggered mitochondrial translocation in mast cells, which is a critical process accompanying mast cell degranulation. Moreover, the two flavones decrease NF-κB activation, and we report for the first time to our knowledge that, they also inhibit NF-κB induction at the transcriptional level. By doing so, luteolin and methoxyluteolin could effectively block the activation of both mast cells and keratinocytes, reduce infiltration of other immune cells, and limit inflammation. Lastly, the two flavones decrease keratinocyte proliferation without affecting intracellular energy production. Hence, luteolin and methoxyluteolin have the potential to be developed into safer and more effective alternative therapies for the treatment of inflammatory skin diseases including Ps, which is characterized by keratinocyte hyperproliferation, mast cell activation and chronic inflammation.

Figure 5.1. Diagrammatic representation of inhibitory actions of luteolin and methoxyluteolin on mast cell and keratinocyte activation.



It is important to note that luteolin and methoxyluteolin inhibit human mast cell activation much more effectively than cromolyn does, which is the only clinically available "mast cell stabilizer". Luteolin and methoxyluteolin are also more potent than the flavonol quercetin. Increasing evidence indicate that mast cells are widely distributed throughout the body and actively interact with other cell types in their surrounding environment, thereby playing a critical role in virtually every tissue, through participation in the induction and/or propagation of various immune and inflammatory responses. Thus, the inhibitory actions of luteolin and methoxyluteolin on mast cells are not only important for Ps, but can also be applied to a board spectrum of inflammatory conditions, including atherosclerosis, asthma, autism, diabetes, interstitial systitis, multiple sclerosis, obesity, as well as certain types of cancer[7, 44, 45, 252, 253].

Due to extensive metabolism, plasma concentrations of quercetin and luteolin aglycones in humans on a normal diet do not exceed 10 μM[167, 168]. In our studies, luteolin and methoxyluteolin effectively inhibit activation of mast cells and keratinocytes at concentrations as low as 10 μM, which could be achieved *in vivo* after the use of supplements. On the other hand, the effects of the two flavones at concentrations of >10 μM may not be physiologically valid. However, higher *in vivo* concentrations could be obtained in the intestinal lumen. In addition, deconjugation can potentially occur *in vivo* to convert flavonoid metabolites back to aglycones in certain tissues, leading to reactivation of the conjugated flavonoid[254-256]. Moreover, circulating metabolites of quercetin and luteolin may also have biological actions. Hence, flavonoids and their

metabolites can indeed reach effective concentrations in tissues. Taking these into account, our results are physiologically relevant.

In our studies, the most potent compound among various flavonoids tested is the unhydroxylated flavone, methoxyluteolin with 3',4',5,7-tetramethoxyl group substitution, which is expected to have no antioxidant activity. This suggests that flavones may utilize more specific ways to interact with different receptors and/or signaling molecules involved in cellular processes. In fact, our results indicate that the two flavones effectively block intracellular calcium elevation, reduce mitochondrial translocation, decrease induction of the transcription factor NF-κB at both the gene and protein levels, as well as inhibit cellular proliferation. The observed order of inhibitory potency is methoxyluteolin > luteolin > quercetin. The structural variations of these three flavonoids may contribute to the observed differences in their activities. In order to further clarify the structural determinants involved in their biological activities, we searched the literature for the structural substitutions and observed effects of some typical flavonoids, and summarized in Table 5.1. In conclusion, the structure-activity relationships of flavonoids are: (1) the C2=C3 double bond in the center ring is essential for the activity, because the potency of naringenin with a C2-C3 single bond is significantly lower compared to all the other flavonols and flavones. (2) multiple hydroxyl groups at C3', C4', C5 and C7 positions are very important for flavonoid activity, since luteolin and quercetin are more potent than kaempferol, which has one less hydroxyl groups at the C3' position. (3) flavones appear to be more active than flavonols, and the hydroxyl group substitution at the C3 position (as in flavonols) does not confer

higher potency. For example, luteolin, which lacks the C3-hydroxyl group, is more effective than quercetin. (4) methoxyl substitutions at C3', C4', C5 and C7 positions increase potency, as seen with methoxyluteolin and tangeretin. Our findings are significant and indicate that methoxy substitutions at certain sites not only preserve, but also enhance the biological activities of flavonoids. Hence, it is possible that luteolin can have greater inhibitory effect in some tissues and cells, where metabolism produces the methoxylated forms of luteolin.

Table 5.1. Structural substitutions of flavonoids and the relative potency of their inhibitory actions

Inhibitory Potency	Flavonoids		Substitutions						Models used	Biological activity	References
	Subgroup	Name	C2=C3	3'	4'	3	5	7			
Highest	Flavone	Methoxyluteolin	Yes	OCH_3	OCH_3	H	OCH_3	OCH_3	Human mast cells and keratinocytes	Anti-inflammatory, antiproliferative	Present study
		Tangeretin	Yes	H	OCH_3	H	OCH_3	OCH_3	Mice	Reduce scratching	[257]
									Hamsters	Lipid-lowering	[258]
									Colorectal cancer cells	Antiproliferative	[259]
		Diosmetin	Yes	OH	OCH_3	H	OH	OH	Mouse ear edema model	Anti-inflammatory	[260]
									Mice	Anticancer	[261]
									Rat mast cells	Mediator release	[262]
									Mouse macrophages	COX-2 expression	[262]
		Luteolin	Yes	OH	OH	H	OH	OH	Human mast cells and keratinocytes	Anti-inflammatory, antiproliferative	Present study
									Prostate xenograft mouse model	Anticancer	[263]
									Rat mast cells	Mediator release	[264]
									Mouse macrophages	TNF release	[265]
	Flavonol	Quercetin	Yes	OH	OH	OH	OH	OH	Mouse macrophages	TNF release	[265]
									Rat mast cells	Antioxidant	[264]
									Human mast cells	Mediator release	Present study
Lowest	Flavanone	Naringenin	No	H	OH	H	OH	OH	Human HMC-1 mast cells	Mediator release	[266]
									Rat mast cells	Antioxidant	[264]

* All publications did not compare all flavonoids in all systems.

Currently, more toxicological data are needed to prove bioavailability and safety of flavonoids in humans, before clinical investigations can be carried out. Recently, a few studies have reported antiestrogenic (and estrogenic) actions of flavonoids on the "hormonal system". For example, quercetin up-regulates gene expression of estrogen receptors α and β in human hepatocytes[211]. Luteolin has been shown to inhibit aromatase and decrease estrogen biosynthesis in human ovarian granulosa cells[267]. The conclusions made from these studies have since raised concerns about the use of flavonoids. In particular, these authors concluded that flavonoids may alter sex differentiation and increase the risk of reproductive tract cancers or developmental disorders[268]. However, these studies were carried out using transformed cell lines *in vitro*, which do not represent the true "hormonal system". Additionally, no data exist on the effects of flavonoids administered as pure compounds to humans[155, 269]. Finally, plasma concentrations after oral administration of these flavonoids would be 10-100 times lower than the concentration reported to have endocrine disrupting effects[268]. Therefore, flavonoids are considered to have very weak hormonal effects.

Based on the inhibitory effects of luteolin and methoxyluteolin on inflammatory mediator release, intracellular calcium elevation, mitochondrial translocation and NF-κB activation in human mast cells and keratinocytes, as well as anti-proliferative actions as described here, luteolin and methoxyluteolin are the best flavonoid candidates for treating inflammatory diseases *in vivo*. The promising benefits of natural flavonoids deserve further research with well-designed studies using *in vivo* models and clinical trials on appropriate patient populations. In fact, a number of pilot clinical trials using quercetin,

luteolin and flavonoid combinations have yielded significant benefit in chronic prostatitis[270], in bladder pain syndrome/interstitial cystitis[271-273], and in autism[176, 274, 275]. Hence, there are already considerable safety and efficacy preliminary results on clinical applications of natural flavonoids.

A major drawback of the use of cromolyn in the clinical setting is the poor water solubility. Luteolin and methoxyluteolin are even more difficult to dissolve in water. Because flavonoids are mainly targeted for disease prevention, oral administration is the appropriate route, except for treating Ps where luteolin and methoxyluteolin could be developed into cream or ointment formulations for topical application, which could achieve high concentrations locally. Flavonoids have typical oral bioavailability of 10% or less in animals and humans[167]. Limitations of their clinical efficacy due to low oral bioavailability may be overcomed by utilizing novel drug delivery systems. A liposomal or enteric coated formulation may offer advantage for increased water-solubility and hence improved bioavailability and prolonged blood circulation after oral administration, which has been shown for quercetin[276]. In addition, encapsulating quercetin in novel nanoparticles coated with targeting ligand not only greatly enhanced its oral absorption but also actively delivered quercetin to tumor sites[277]. Similar encapsulation and formulations with nanotechnology may be applied to luteolin and methoxyluteolin. Furthermore, these two flavones could be formulated with the natural olive kernel oil for better oral absorption. Olive kernel oil is also rich in flavonoids, which is likely to offer additional benefit when combined with luteolin and methoxyluteolin.

Taken together, the present study not only demonstrates the potent inhibitory actions of luteolin and its analogue methoxyluteolin on human mast cell and keratinocyte activation, but also provides more information on their mechanisms of action. Our results further illustrate the structure-activity relationship of flavonoids, and indicate that methylation on certain sites enhances the inhibitory actions of luteolin. With the help of novel formulations that improve oral absorption, the successful development of luteolin and methoxyluteolin as safer and more effective anti-allergic, anti-inflammatory and anticancer agents in the future will soon be within our reach.

Future Directions

Our results indicate that flavones decrease activation of the transcription factor NF-κB. More studies are required to investigate effects of flavones on signaling molecules upstream of NF-κB activation, such as PI3K, Akt, and the mammalian target of rapamycin (mTOR). The mTOR complex 1 (mTORC1) kinase is an important component of PI3K/Akt signaling pathway which governs cell size, growth, and metabolism[278]. Recent papers provided early evidence that the mTOR pathway may be involved in activation and proliferation of mast cells [279, 280] and keratinocytes[281, 282]. For instance, activation of mTORC1 leads to increased mast cell proliferation and function[280], and differentiation[283]. Moreover, toll-like receptor stimulation activates NF-κB and cytokine production through mTOR in human oral keratinocytes[284]. Another recent paper further reported that keratinocyte proliferation is regulated by PI3K/Akt/mTOR signaling cascade[285]. Thus, it is interesting to determine if luteolin and methoxyluteolin have any effects on these signaling molecules.

Treatment with luteolin or methoxyluteolin effectively inhibits activation of human mast cells and keratinocytes. We should also try incubating cells with these two flavones in combination, in order to study if they have any additive, synergistic or antagonizing effects. Due to the structural similarities between luteolin and methoxyluteolin, these two flavones are likely to have similar mechanisms of actions, and therefore potential antagonizing effects when used in combination. However, they could also act through different signaling pathways and exert additive or even synergistic effects when applied together. If this is the case, then the two flavones can be developed

in combination therapy with a lower concentration for each flavone, which would also decrease any potential adverse reactions. Further experiments are needed to uncover these effects.

In our studies, we examined effects of luteolin and methoxyluteolin on pure cultures of human mast cells and keratinocytes separately. It would be interesting to co-culture these two cell types, which better represent the interactions between mast cells and keratinocytes in the skin under physiological conditions. Experiments should be carried out to establish a long-term co-culture model that includes dermal mast cells, fibroblasts and keratinocytes, which would act as a more relevant tool to allow further investigation on the interactions between major skin cells and potential therapeutic effects of luteolin and methoxyluteolin for treating inflammatory skin conditions.

In addition, it would be interesting to determine whether other methoxy-analogues of luteolin are equally effective as methoxyluteolin in blocking mast cell and keratinocyte activation. Some examples would be 3'-monomethoxyluteolin, 3',4'-dimethoxyluteolin, 5,7-dimethoxyluteolin, 3',4',5-trimethoxyluteolin. Evidence from previous studies indicates that increasing number of methoxy substitutions are correlated with enhanced inhibitory actions of luteolin (Table 5.1). However, it is possible that methoxy substitution on certain sites diminishes the inhibitory actions of luteolin, while methoxy substitution on some other sites super enhances its inhibitory actions. If this is the case, combining different methoxy substitutions might show no improvement on inhibitory

activity of luteolin due to masking effects. Results from these comparisons would shed more light on structure-activity relationships of flavonoids on their inhibitory actions.

One shortcoming of our study is the use of pure, unmetabolized flavone aglycones. Under physiologically relevant conditions, flavonoids are exposed to extensive metabolism to form methyl, sulfate and glucuronate conjugates of the parent compounds,which are likely to possess different biological properties and distribution patterns within tissues and cells than do pure flavonoid aglycones. Therefore, *in vitro* experiments with the form of pure aglycones are not necessarily relevant to the *in vivo* situation. It is necessary to further determine the various metabolites of luteolin and methoxyluteolin, and investigate whether these metabolites are biologically active as compared to their parent compounds.

A recent study had reported that luteolin and quercetin increased the expression and secretion of the anti-inflammatory cytokine IL-10 at low concentrations (1-10 μM) in mouse bone marrow-derived macrophages. However, this IL-10-inducing effect disappeared with increasing concentrations of both flavonoids, and luteolin actually inhibited IL-10 expression at 100 μM[265]. This interesting finding suggests two potential aspects that are worthwhile to further investigate: (1) flavonoids may help resolve inflammation by inducing IL-10 production. (2) flavonoids could be pro-oxidative at high concentrations. More research studies in this aspect are needed to help us understand the precise mechanisms involved.

In order to develop flavones into clinical agents, more pharmacokinetic and toxicology data are needed to prove their bioavailability and safety before clinical investigations can be further carried out in humans. It is also necessary to determine pharmacokinetic profiles and potential toxicity of luteolin and methoxyluteolin in mice or rats administered by oral gavage. Another important issue is that flavones may influence the absorption, metabolism and disposition of other drugs by interacting with various efflux transporters and metabolizing enzymes, such as the breast cancer resistance protein and cytochrome P450 enzymes. In humans on a balanced diet, the plasma level of flavonoids usually ranges between nanomolar to low micromolar concentrations due to their poor oral bioavailability, which is far below the concentrations tested under *in vitro* conditions. However, after the use of dietary supplements, higher flavonoid plasma concentrations may be obtained, especially in the colon. Moreover, in humans, large interindividual variability can occur in drug-metabolizing enzymes as a result of genetic and environmental factors. We believe more studies in this area of research should be conducted with physiologically relevant concentrations of luteolin and methoxyluteolin, which would help us unveil the adverse effects and potential drug-drug interactions of these two flavones.

Once the pharmacokinetic and toxicology profiles of luteolin and methoxyluteolin have been established, we can then investigate their biological efficacies by using mouse models for inflammatory diseases. For example, we can use the *scid/scid* CD4+CD45RB^{hi} T cell immune transfer model for Ps, because it best resembles the clinical and histopathological features of human Ps and responds to many clinically

available treatments for Ps[286]. Luteolin and methoxyluteolin could be administered either *ip* or by oral gavage (before or after the initiation of Ps-like symptoms), or as a skin ointment applied after the first appearance of Ps lesions. Mice skin Ps lesions can be monitored before and after flavone application, as well as expression of various inflammatory molecules and biomarkers using qRT-PCR and immunohistochemistry.

Additional work related to my thesis:

Chapter 6. Luteolin Inhibits IL-8 Production in Human Melanocytes

Introduction

Vitiligo is a cutaneous disorder of de-pigmentation affecting 1-3% of the general population[287], characterized by absence of functional melanocytes. The exact pathogenesis of vitiligo remains obscure. Recent evidence suggests that oxidative stress, autoimmune responses and melanocyte apoptosis are involved[287].

The release of inflammatory cytokines, especially IL-1, IL-6 and TNF after skin trauma (Koebner phenomenon) might lead to the recruitment of T cells in the skin and exposure to new antigen-expressing melanocytes. Th-1 cells can increase angiogenesis by inducing the expression of VEGF in both Th-1 cells and mast cells[288]. TNF has been shown to induce IL-8 mRNA expression in a melanoma cell line [289] and to upregulate IL-8 receptor expression in normal melanocytes[290]. IL-8 is a chemokine important in inflammatory skin diseases and is produced by monocytes, mast cells, fibroblasts, endothelial cells, dendritic cells and keratinocytes[291]. IL-8 is chemotactic to neutrophils, T-lymphocytes, basophils and keratinocytes[291].

Here we studied the effect of TNF and IL-1β on IL-8 release from human cultured primary melanocytes, before and after treatment with the natural flavone luteolin.

Methods

Human primary melanocytes were obtained from ATCC (Manassas, VA, PCS-200-013) and cultured in Dermal Basal Cell Medium (ATCC, PCS-200-030) supplemented with 100 U/mL penicillin/streptomycin and 50 μL phenol red (ATCC, PCS-999-001).

Melanocytes (5×10^4 cells per well) were seeded in 12-well plates (Becton Dickinson) and allowed to grow overnight before stimulation with either TNF (100 ng/mL) or IL-1β (10 ng/mL) for 24 h. In some experiments, cells were pretreated with luteolin (50 μM, 30 min), while control cells were treated with DMSO (0.1%). IL-8 was measured in supernatant fluids by ELISA using a commercial kit from R&D Systems.

Experiments were performed in triplicates, and were repeated at least three times (n=3). Results on IL-8 release were analyzed using Student's *t*-test. Data are expressed as mean ± SD. Significance is denoted by *$p<0.05$ and **$p<0.01$.

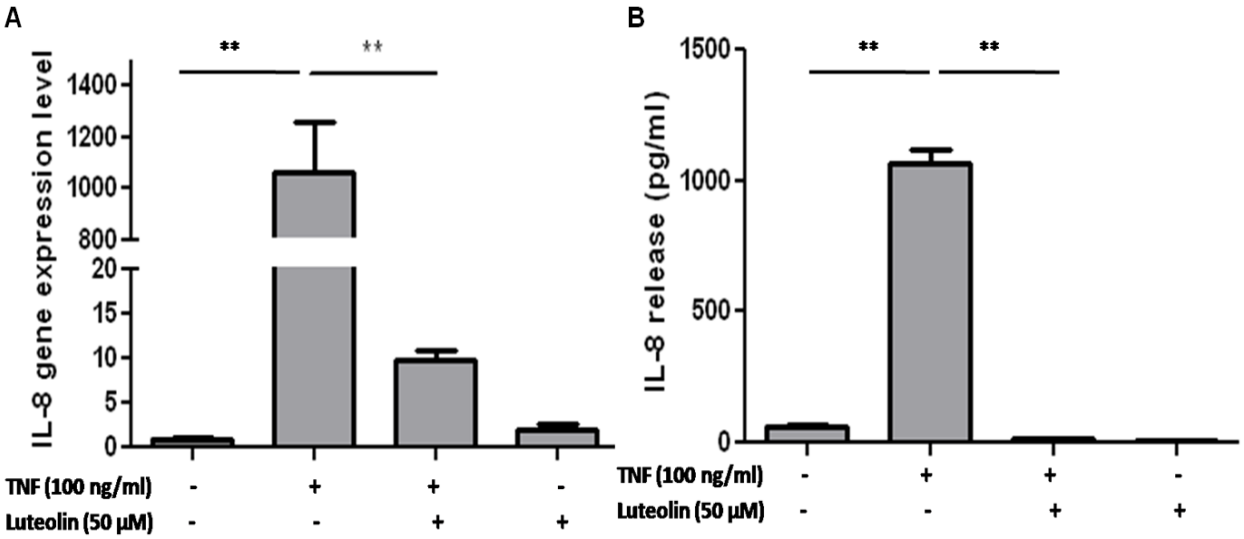
Results

Luteolin decreases TNF-triggered IL-8 production in human melanocytes

Incubation of melanocytes with TNF (100 ng/mL, 24 h) induces statistically significant ($p<0.01$) IL-8 gene expression (Fig. 6.1 A) measured by qPCR, and release (Fig. 6.1 B) measured by ELISA ($p<0.01$). Incubation of melanocytes with luteolin (50 μM, 30 min) prior to stimulation with TNF (100 ng/mL) significantly inhibits ($p<0.01$) both melanocyte IL-8 gene expression (Fig. 6.1 A) and release (Fig. 6.1 B).

Figure 6.1. Effect of luteolin on TNF-induced IL-8 production in human melanocytes

IL-8 gene expression (A) and release (B) from cultured melanocytes with or without pretreatment with luteolin (50 μM) for 30 min, before stimulation with TNF (100 ng/mL) for 24 h (n=5, ** $p<0.01$).

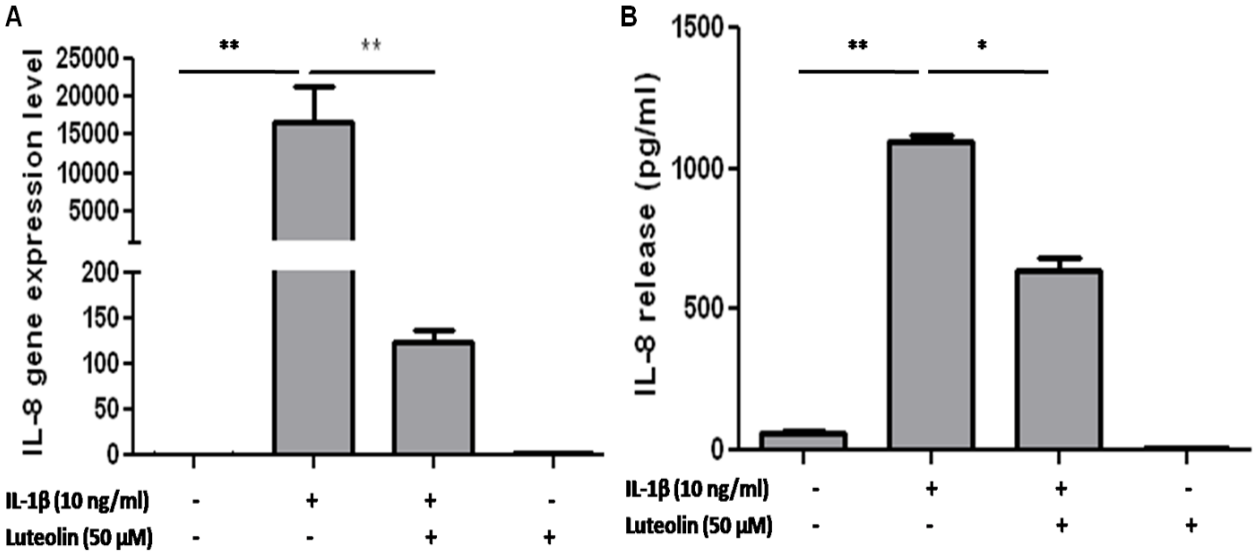


Luteolin decreases IL-1β-triggered IL-8 production in human melanocytes

Incubation of melanocytes with IL-1β (10 ng/mL, 24 h) induces statistically significant ($p<0.01$) IL-8 gene expression (Fig. 6.2 A) and release (Fig. 6.2 B) in the supernatant fluid. Preincubation with luteolin (50 μM, 30 min) prior to stimulation with IL-1β significantly inhibits both melanocyte IL-8 gene expression ($p<0.01$, Fig. 6.2 A) and release ($p<0.05$, Fig. 6.2 B).

Figure 6.2. Effect of luteolin on TNF-induced IL-8 production in human melanocytes

IL-8 gene expression (A) and release (B) from cultured melanocytes with or without pretreatment with luteolin (50 μM) for 30 min, before stimulation with IL-1β (10 ng/mL) for 24 h (n=5, * $p<0.05$, ** $p<0.01$).



Discussion

This is the first report to our knowledge showing increased release of IL-8 from human melanocytes in response to TNF and IL-1β. We propose that in the initial stages, where melanocytes are still present in the epidermis, IL-6, IL-1 and TNF released by various cell types [291] could stimulate high IL-8 skin expression by melanocytes. IL-8 is a powerful chemokine that has been reported to induce oxidative stress, leading indirectly to both keratinocyte and melanocyte apoptosis in vitiligo[287]. Apoptotic cells could in turn release high amounts of proinflammatory IL-1β and TNF, further enhancing skin inflammation. We previously showed that both preformed and newly synthesized TNF can be released from mast cells[31], which appear to be increased in the center of vitiligo lesions[288]. A recent paper reported that chemically-induced vitiligo led to increased production of IL-6 and IL-8[292].

We also report for the first time that the natural flavone, luteolin, blocks TNF- and IL-1β-induced IL-8 gene expression and release from human melanocytes. Luteolin is a safe, plant-derived polyphenolic compound with antioxidant, anti-inflammatory, anti-allergic and cytoprotective properties[147]. Moreover, the structurally related flavonol to luteolin, quercetin, was reported to induce melanogenesis in a human epidermal culture model[293].

Our results suggest the involvement of IL-8 in the initial vitiligo stages and a potential protective role of luteolin in IL-8-induced inflammation.

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APPENDIX

Publications

1. **Weng Z***, Zhang B*, Asadi S, Sismanopoulos N, Butcher A, Fu X, Katsarou-Katsari A, Antoniou C, Theoharides TC. Quercetin is more effective than cromolyn in blocking human mast cell cytokine release and inhibits contact dermatitis and photosensitivity in humans. ***PLoS ONE.*** 2012; 7(3):e33805.

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