The anti-inflammatory effects of platinum nanoparticles on the lipopolysaccharide-induced inflammatory response in RAW264.7 macrophages

RAW264.7マクロファージにおけるLPS誘導炎症反応に対する
白金ナノ粒子の抗炎症効果

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Abbreviations:

Nano-Pt: Platinum nanoparticles

RT: Reverse transcription

PGE_2: Prostaglandin E_2

iNOS: inducible nitric oxide synthase

MAPK: Mitogen activated protein kinases

NO: Nitric oxide

ROS: Reactive oxygen species

AD: Atopic dermatitis

CVD: Cardiovascular disease

TLR: Toll like receptors

TNF: Tumor necrosis factor

LPS: Lipopolysaccharide

ROI: Reactive oxygen intermediate

MCP-1: Monocyte chemoattractant protein

COX-2: Cyclooxygenase-2

RHD : Rel homology domain

IKB-α: Inhibitor kappa B alpha

ERK : Extracellular signal-regulated kinase

JNK: c-Jun NH2 terminal kinase

SOD: Superoxide dismatuse

CAMs: Classical activated macrophages

AAMs: Alternatively activated macrophages

NF-$_\kappa$B: Nuclear factor kappa B

Chapter 1

General Introduction

Part 1

1.1 Inflammation

Inflammation is a highly complex and highly regulated biological process, which involves the cascade of events initiated to defend against the foreign challenge or tissue injury (1, 2). Inflammation which is characterized by edema, pain, redness, and heat, is one of the most important elements that protect body from the invading pathogens such as bacteria or viruses (3). Two main types of inflammation have been identified, depending on the duration of inflammatory response; (i) acute inflammation, lasts for shorter period of time ranging from minutes to few days and (ii) chronic inflammation, lasts for longer period of duration (2).

Increased vascular permeability, exudation, edema and infiltration of leukocytes are characteristics of acute inflammation, while chronic inflammation is characterized by infiltration of lymphocytes and macrophages with angiogenesis, fibrosis and tissue necrosis at sites of inflammation (4, 5). The inflammatory response must be tightly regulated because aberrant regulation of inflammation has been considered as one of the leading cause for increasing human cancers (6). The key molecules that play an important role in the regulation of inflammatory response include active species like nitric oxide (NO) and reactive oxygen species (ROS,

superoxide anion, hydrogen peroxide, and hydroxyl radical), additionally enzymes such as inducible nitric oxide synthase (iNOS), which is well known as an index of inflammation. Overproduction of these key molecules causes excessive inflammatory response (7, 8).

1.1.1 Inflammation and Cancer

The association between inflammation and cancer is not new. It was in the year 1863 when Rudolf Virchow explained the association of chronic inflammation with cancer. He suggested that the presence of “lymphoreticular infiltrate” reflects the origin of cancer at sites of chronic inflammation (9, 10). About 15% of the malignancies around the globe can be attributed to infectious agents and inflammation is one of the major components of these chronic infections **(Table 1)**. Chronic inflammation is caused by persistent infections inside the host. DNA damage in the proliferating cells was induced by leukocytes and other phago-cytic cells by the generation of reactive oxygen (ROS) and nitrogen species that are normally produced by these cells in order to fight infections (10, 11).

Table 1. Association between Inflammation and Cancer

Inflammatory stimulus /Pathologic condition	**Associated neoplasms/Malignancy**
Asbestosis, silicosis	Mesothelioma, Lung carcinoma
Bronchitis	Lung carcinoma
Cystitis/Inflammation of bladder	Bladder carcinoma
Gingivitis, Lichen planus	Oral squamous cell carcinoma
Inflammatory bowel disease, Crohn's disease, Chronic ulcerative colitis	Colorectal carcinoma
Lichen sclerosus	Vulvar squamous cell carcinoma.
Chronic pancreatitis, Hereditary pancreatitis	Pancreatic carcinoma
Reflux oesophagitis, Barrett's oesophagus	Oesophagal carcinoma
Sialadenitis	Salivary gland carcinoma
Sjogren syndrome, Hashimoto's thyroiditis	MALT lymphoma
Skin inflammation	Melanoma

Modified from ref. **(9, 10)**

1.1.2 Inflammation and Chronic Diseases

Chronic inflammation has also been implicated in the pathogenesis of several chronic diseases which includes cardiovascular disease (CVD) (12), obesity (13), diabetes (14) and chronic inflammatory skin diseases such as psoriases and atopic dermatitis (AD) (15).

Atopic dermatitis is one of the most frequent chronic inflammatory conditions with an increasing prevalence rate of 10% - 20% in children and affects 1% - 3% of adults in industrial countries (16, 17). It impairs the patient's quality of life and their families, and causes the economic burden which has been estimated to be billions of dollars (18).

1.2 Role of Macrophages in Inflammation

Macrophages are the key cells that play an important role in the inflammation. During the inflammatory process, these phagocytic cells become activated and have destructive effects. Usually the body protects the entry of foreign antigens by the natural physical barriers like skin and mucosal membranes of the innate immune response. Besides providing a barrier, the skin contains many immune cells that can be activated by invasion of pathogens or skin damage. Macrophages are one of the most crucial

immune cells involved in inflammation and exhibits different immunological functions in the skin (16, 19).

Macrophages performed different functions, in case the antigen get pass through the physical barrier, macrophages and neutrophils migrate to the site of antigen and engulf, phagocytize the invading microorganism, and also the debris, left behind after cell destruction. Another important function perform by macrophages is the expression of varied proteins or enzymes, reactive oxygen species (ROS) production, to regulate many other cells through the release of cytokines and chemokines. Macrophages also play their role in the presentation of antigen to T lymphocytes for activation of cellular immunity (19, 20). Macrophages can be differentially activated on the bases of their gene expression profile in response to specific stimuli. The gene-expression-based phenotyping of macrophages is classified as ***classical activated macrophages*** **(CAMs)** and ***alternatively activated macrophages*** **(AAMs)**. The classically activated macrophages are also called M1 macrophages. These macrophages are activated on exposure to following signals: toll-like receptor (TLR) stimulation, bacterial infection, and interferon- (IFN-) γ alone or with microbial products [lipopolysaccharide (LPS)] or cytokines [tumor necrosis factor (TNF)] (19, 21). Therefore, signals from (IFN-) γ , TNF or LPS are essential for the classical activation

of macrophages, release of high production of toxic intermediates [nitric oxide (NO) and reactive oxygen intermediates (ROI)] has been associated with these classically activated macrophages (21, 22).

Alternatively activated macrophages (AAMs) are also known as M2 macrophages. IL-4 and IL-13 signals are associated with the activation of AAMs macrophages. AAMs have been further classified into three sub-types such as M2a, M2b and M2c, depending on the functions performed by different types of macrophages in varied homeostatic, infectious, tissue repair and regeneration conditions (23, 24) Hence, it is become clear that there are not only few activation states for macrophages but a variety of responses are involved depending on the individual stimuli.

Macrophages are widely distributed throughout the body, and play a crucial role in the pathogenesis of several inflammatory diseases. Therefore, necessary step should be taken to develop new methods to reduce the activated macrophages and to inhibit macrophage products that take part in inflammation and in various inflammatory diseases. As described above macrophages can be activated by different stimulus. ***Lipopolysaccharide (LPS)***, a structural component of the outer membrane of Gram-negative bacteria, is one of the most potent activators of macrophages (25). LPS induced activation of macrophages caused different responses, which includes secretion

of growth factors, release of pro-inflammatory mediators, phagocytosis, and cyto-skeletal rearrangement (26). LPS, binding to its membrane receptor, TLR4, activates the gene transcription and enhance the inflammatory response by the production of pro-inflammatory cytokines, including tumor necrosis factor-α (TNFα), interleukins such as (IL)-1β, IL-6, monocyte chemoattractant protein (MCP-1) and other inflammatory mediators like NO, ROS and cyclooxygenase-2 (COX-2) in macrophages (20,27). Therefore, the inflammatory response has been extensively studied in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells, because this cell line is very sensitive to LPS stimulation, provides an excellent model for the screening of anti-inflammatory drugs and helps to evaluate the inhibitors of pathways that are involved in the induction of cytokines and pro-inflammatory enzymes (28,29,30). Further, investigation of these pathways may help us to develop new anti-inflammatory drugs or promote the therapeutic strategy for inflammation and inflammatory diseases.

1.3 Oxidative Stress and Inflammation

Reactive oxygen species (ROS) can be generated by different cellular sources within a cell (Fig.1). Which were further divided into two main groups. In one group, ROS release as a byproduct or waste product in response to various biological processes.

In another group, ROS are generated intentionally as signal transduction molecules or as cell defense molecules such as in inflammation (31).

Mitochondria are the measure intracellular source of ROS, due to the leakage of electron during transfer between electron transport chain complexes. This leakage of an electron facilitates the reaction with molecular oxygen and resulted in the generation of ROS. Electron leakage and resulting production of ROS has been implicated as the byproduct but recent studies have mentioned the importance of this ROS generation in cell and tissue injury (32, 33). The second main ROS sources are enzymes that generate ROS for different biological purposes, which include phagocytic NADPH oxidase, xanthine oxidase, cytokines and various growth factor receptors (34, 35).Molecular oxygen reduce to superoxide by the action of NADPH oxidase, a family of transmembrane oxidases. This reaction takes place due to the consumption of energy produced from the oxidation of NADPH/NADH to NADP/NAD (36). This superoxide plays an important role in the defense against various infectious pathogens (37).

Aberrant regulation of ROS can caused oxidative modification of proteins, lipids and DNA (38). Many researchers have found that the generation of mitochondrial ROS enhances the inflammatory response, involved in signaling and cancer progression (39, 40). Therefore, the toxic buildup of ROS is regulated by the balanced system of

enzymes such as superoxide dismutases, catalase, thioredoxins and glutathione reductase (41, 42). However, the abnormal ROS accumulation or anti-oxidant defense failure, have been implicated in the activation of mitogen-activated protein kinase (MAPks), inflammatory transcription factors (43), NF-$_{\kappa}$B signaling (44), which all play crucial role in the regulation of cell proliferation, metabolism, survival and in cancer development.

Macrophages are the cells that produce and release ROS in response to phagocytosis and various stimuli like activation with LPS. The LPS induced overproduction of ROS by macrophages induces oxidative damage to membrane lipids, DNA, proteins and lipoproteins. As mentioned earlier ROS also has been involved in the modulation of NF-$_{\kappa}$B activation. Thus, ROS inhibition is a well-known therapeutic target in the treatment of several inflammatory diseases.

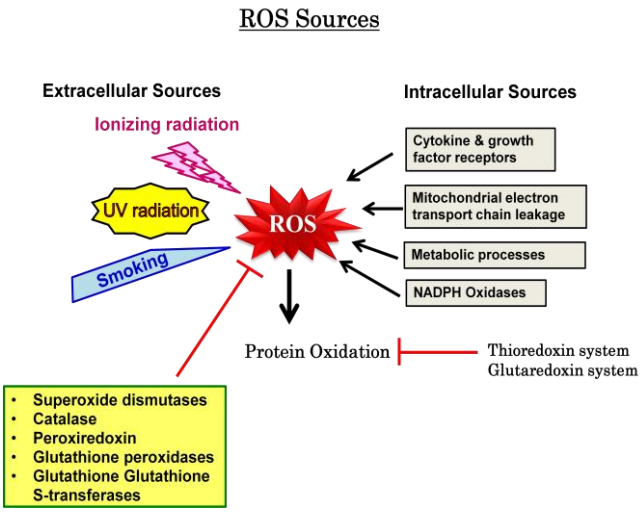


Fig.1 Sources of ROS. (modified from ref. [31])

1.3.1 Nitric Oxide

Nitric oxide (NO) has been the focus of intense research for its role in different biological and pathological processes. NO was first discovered as a mediator of relaxing factor for smooth muscles, since then it was found that NO has been engaged in the variety of different physiological processes (45, 46). NO is an inorganic molecule synthesized from the conversion of L-arginine to L-citrulline by the action of enzyme, nitric oxide synthetase (NOS). There are three isoforms of NOS:

(i) Neuronal NOS (nNOS or NOS1): mainly expressed by neurons in the brain and enteric nervous system.

(ii) Endothelial NOS (eNOS or NOS3): expression of this isoform confined to endothelial cells lining the vasculature (45, 47). Both eNOS and nNOS enzymes are cytosolic and calcium/calmodulin dependent, and produce NO in low amounts for a short period of time depending upon the stimulation. Low amounts of eNOS and nNOS has been regarded as beneficial for many physiological responses, including neutrotransmission, vascular homeostasis and host defense (48, 49).

(iii) Inducible NOS (iNOS or NOS2): The third isoform of NOS is calcium independent and produce NO for prolonged periods of time. Increase production of NO by iNOS is harmful and involved in many physiological and pathological processes such as inflammation and tumorigenesis (45, 49). Macrophages express iNOS on activation by a cytokine and bacterial elements like LPS and IFN-γ. In activated macrophages, the transcriptionally expressed iNOS is responsible for the prolonged and profound NO production (47, 50). Therefore, inhibition of NO production by blocking iNOS expression is known as an important strategy in the treatment of inflammatory diseases.

1.4 Role of NF-$_\kappa$B in Inflammation

Nuclear factor kappa B (NF-$_\kappa$B) was first identified as a transcriptional regulator of the expression of immunoglobulin light chain gene (51, 52). NF-$_\kappa$B is an important transcription factor due to its central role in inflammation, immunity, cellular activities, including cell proliferation, cell death and control of genes that are involved in biological processes (53, 54). NF-$_\kappa$B factors are expressed in almost all mammalian cells, NF-$_\kappa$B proteins consist of well-known five different family members: Rel A (p65), RelB, c-Rel, p50 and p52 that can bind to, homodemerize and heterodimerize depending

on their Rel homology domain (53, 54, and 55). All of these members possess a highly conserved N-terminal region, known as Rel homology domain (RHD), which is essential for DNA binding and dimerization. All NF-$_{\kappa}$B proteins can combine to form homodimers and heterodimers with other NF-$_{\kappa}$B proteins, excluding RelB, which only forms heterodimers (55, 31). The usually activated form of NF-$_{\kappa}$B is a heterodimer, which consist of most common dimer complex p50/RelA and is play an important role in inflammatory and innate immune responses. Under normal conditions NF-$_{\kappa}$B is retained in the cytoplasm in an inactive form by a family of inhibitors (IkBs). A small inhibitory molecule, inhibitor kappa B alpha ($I_{\kappa}B$-α), binds to NF-$_{\kappa}$B and prevents it from entering to nuclei (31, 56) Upon cells stimulation, IkB kinase (IKK) activation, leads to the phosphorylation and degradation of $I_{\kappa}B$-α. The phosphorylated $I_{\kappa}B$-α, released from the complex and cannot retained NF-$_{\kappa}$B in the cytoplasm in its inactive form. The release of NF-$_{\kappa}$B from $I_{\kappa}B$-α results in the subsequent translocation into the nucleus, and activate the transcription of target genes involved in the inflammation (Fig.2).

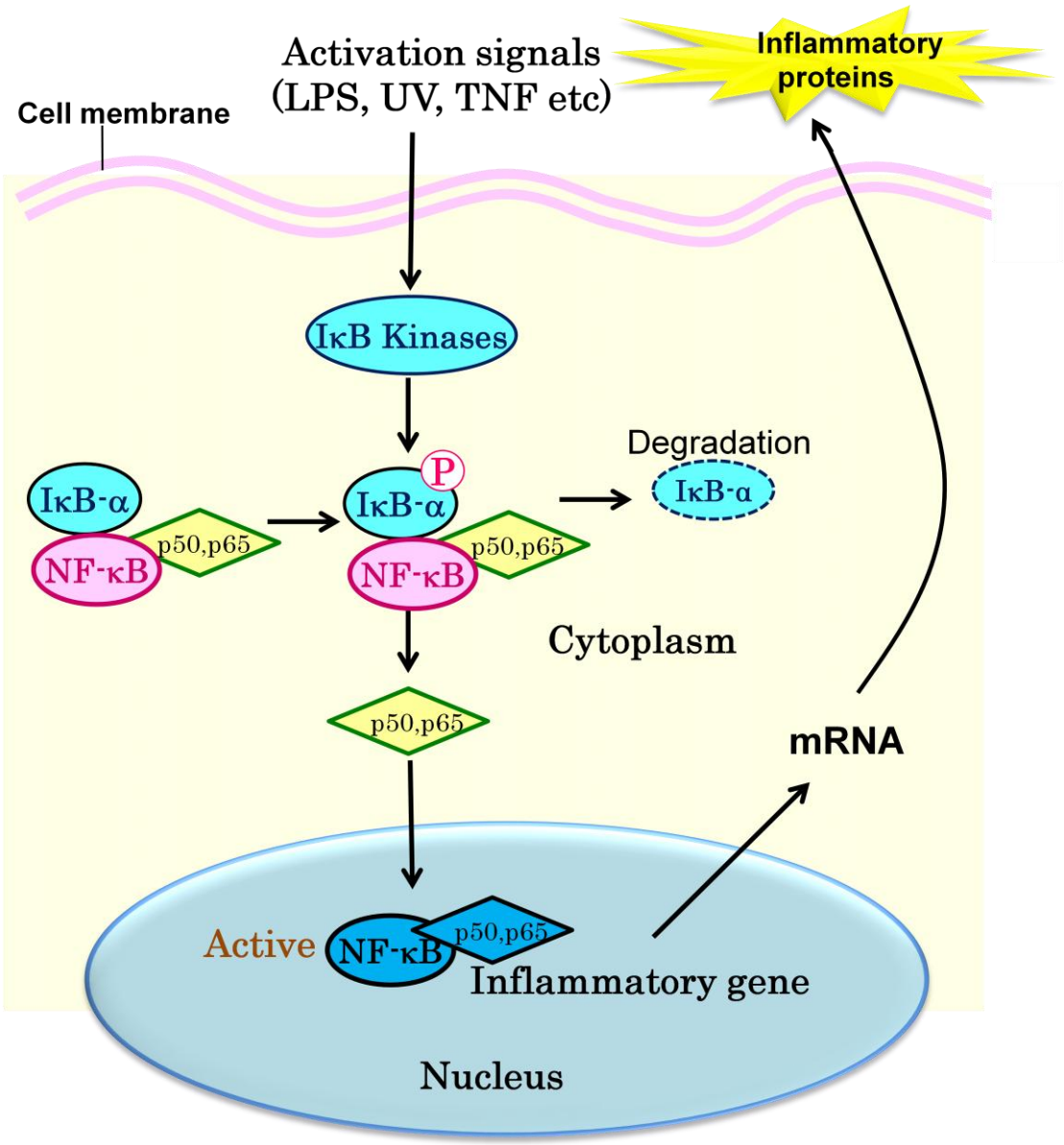


Fig. 2 Schematic Diagram of NF-$_{\kappa}$B activation (modified from ref. [62])

NF-$_{\kappa}$B can be activated by different ways but the two main signaling pathways that lead to the activation of NF-$_{\kappa}$B are: ***canonical (or classical) and non-canonical (or alternative)*** pathways. The ***canonical*** NF-$_{\kappa}$B pathway activation is due to the stimulation of pro-inflammatory receptors such as TNF receptor family, the Toll like receptor family (TLRs) and cytokine receptors. This classical signaling pathway is dependent on IKK complex comprising of IKK-α and IKK-β, which are catalytic kinases, and IKK γ (also referred as NEMO), which acts as a regulatory sub-unit (53,54,57,58). This pathway is usually activated in response to translocation of p50/p65 heterodimer, which plays an important role in inflammation.

The ***non-canonical (or alternative)*** pathway, is mainly stimulated by specific ligation with the family members of TNF receptors, which include LTβR, CD40, BAFF-R and others, mediated by NF-$_{\kappa}$B inducing kinase (NIK). This alternative pathway is strictly dependent on IKKα, its phosphorylation triggers the processing of p100 resulting in the release of p52 (59, 60). P52 binds to its hetero or homo-dimeric partner and able to enter in the nucleus.

Several stimuli have been shown to activate NF-$_{\kappa}$B, including cytokines, bacterial products, viruses and oxidative stress **(Table 2)**. NF-$_{\kappa}$B regulates the expression of several genes for different cytokines and enzymes involved in the immune

and inflammatory responses like inducible nitric oxide synthase and cyclooxygenase-2 (COX-2), which enhanced the production of prostaglandins and thromboxane in inflammatory diseases. NF-$_{\kappa}$B can also be activated in response to the products of gene that are regulated by NF-$_{\kappa}$B such as the pro-inflammatory cytokines interleukin-1β and TNF-α activate and are activated by NF-$_{\kappa}$B (61, 62)

Considerable data has indicated that abnormal activation of NF-$_{\kappa}$B is involved in the pathogenesis of inflammatory diseases and also associated with cancer. Therefore, NF-$_{\kappa}$B is an obvious target for the compounds and drugs with anti-inflammatory activity.

Table 2. Stimuli That Activate NF-κB (modified from ref. [62])

Cytokines	Tumour necrosis factor
	Interleukin-1β
	Interleukin-17
Protein kinase C activators	Phorbol easter
	Platelet-activating factor
Oxidants	Hydrogen peroxide
	Ozone
Viruses	Rhinovirus
	Influenza virus
	Epstein-Barr virus
	Cytomegalo virus
	Adenovirus
Immune stimuli	Phytohemagglutinin
	Anti-CD3 antibodies (by means of T-lymphocyte activation)
	Antigens
Other	Lipopolysaccharide
	Ultraviolet radiation

1.5 Role of MAPKs in Inflammation

Members of the family of mitogen-activated protein kinases (MAPKs) are serione therione kinases that play an important role in intracellular signaling transduction and control major cellular activities, including cell proliferation, differentiation, survival, death and transformation (63, 64). MAPKs activation has been seen in several disease conditions including chronic inflammation and cancer (65, 66). The mammalian MAPK family can be divided into extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2 terminal kinase (JNK), which is also referred as stress-activated protein kinase or SAPK. Each of these were consist of different isoforms: such as ERK (ERK1- ERK8), p38s (p38α, p38*β,* p38 γ , p38 δ), JNK (JNK1,JNK2,JNK3) (64,67, 68).

The three major members of the human MAPK superfamily, which involved in the immune system, are ERK, p38 and JNK. MAPKs are activated due to dual phosporylation on both conserved threonine (Thr) and tyrosine (Tyr) residues, in the regulatory loop (65, 69). Each MAPK group signaling pathway is composed of three components: MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK. Activation of each MAPKs group brought out in a sequential manner, MAPKKK initiates the activation and phosphorylation of MAPKK, which in turn

phosphorylate and activate MAPKs. Once MAPKs are activated they initiate the phosphorylation of several substrate proteins, including transcription factors like EIK-1, c-Jun, ATF2, and p53 (65, 67). The p38 and JNK are known as "stress induced" MAPK that are activated in response to physiological stress, LPS, osmotic stress, oxidative stress, ultraviolet exposure and cytokines like TNF-α and interleukin (IL)- 1β. ERK1/2 pathway is mainly activated in response to stimulation with growth factors (epidermal growth factor, platelet growth factor etc), additionally ERK1/2 is also become activated on exposure to LPS, osmotic stress and adherence in monocytes/macrophages (70, 71, 72).

LPS stimulation in macrophages induced an uncontrolled release of pro-inflammatory mediators, such as TNF-α, ILs, NO and ROS. LPS stimulation activates the transcription factor NF-$_\kappa$B and MAPKs, especially ERK1/2, p38 and JNK. Upon activation MAPKs phosphorylate and control various key cellular activities, including gene expression. MAP kinases ERK, p38 and JNK are known to regulate inflammatory and immune responses and were involved in the LPS induced expression of COX-2 and iNOS in macrophages. Increased expression of COX-2 and iNOS enhanced the production of NO and ROS and the resulting inflammatory response.

Part 2

1.1 Nanomedicine

In recent decades, much attention has been focused on ***nano-science*** and ***nano-technology*.** The concept of nano-scale materials has been widely applied, and it holds promising features for technical, biological, industrial and biomedical applications. Now, nano-technology is rapidly growing field due to increase interest of researcher from academic, industry and federal sector (73, 74). ***Nano-technology,*** deals with the features as small as 1 billion of a meter. The broad application of nano-technology covered almost all fields of life including nanomaterials, nanoscale devices,systems, instruementation research and biomedical research.

The application of nano-technology in the field of medicine is defined as ***nanomedicine,*** which deals with the biology, chemistry, engineering and medicine. It provides help in the use of nanoscale materials and devices for diagnosis and drug delivery, without occluding needles and capillaries, improve disease prevention, diagnosis and treatment of disorders Such as cancer and inflammation (73, 75)

Recently, the use of nanoparticles in the consumer products has been widely increased. The ASTM standard defines nanoparticles as:

"Particles with lengths that range from 1 to 100 nanometers in one or more dimensions" (76).

It has been projected that by 2020 the production of nanoparticles will increase to 58 000 tons (77). Nanoparticles of different metals have been developed and found to catalyze different chemical reaction due to large surface area of smaller particles such as nanoparticles in the form of titanium dioxide and zinc oxide have been used in the sun screens. Nanosilver is widely used in products for its antibacterial effects. Gold nanoparticles have been used as nanoprobes for transmission electron microscope (TEM). Some nobel nanoparticles are reducing catalyst, they may be usable as antioxidants and reduce ROS production in a living body. However, only few studies have shown the antioxidants effects of nanoparticles (78, 79). Nanoparticles possess great effect on environment and human health but in thesis we will mainly focus on the anti-oxidant and anti-inflammatory effects of platinum nanoparticles in mouse murine RAW 264.7 macrophages.

1.2 Anti-oxidant Effects of Platinum Nanoparticles

Recently, platinum nanoparticles (nano-Pt) protected with poly (acrylic acid) were prepared by reducing with ethanol. Platinum nanoparticles has the diameter of 2.0 $\pm$ 0.4 nm (n=200). Nano-Pt are known to act as reducing catalysts, and may possess

antioxidant activity to reduce ROS in living organisms. In support of this notion several researcher has found that these nano-Pt has the ability to scavange ROS, superoxide anion radicals (0_2^-) and hydrogen peroxide (H_20_2), indictaing that nano-Pt can act as SOD/catalse mimetic (80, 81, 82). SOD/catalase mimetics can be co-administered with certain therapeutics to reduce ROS induced damage to normal tissues and protects some of their side effects (83).

Our group has also demonstrated the antioxidant effects of nano-Pt both *in vitro* and *in vivo*. Nano-Pt protects UV induced epidermal inflammation in HaCaT keratinocytes and inhibit hyperthermia induced ROS and apoptosis in human lymphoma U937 cells Fig.3 and 4 (84, 85, 86).

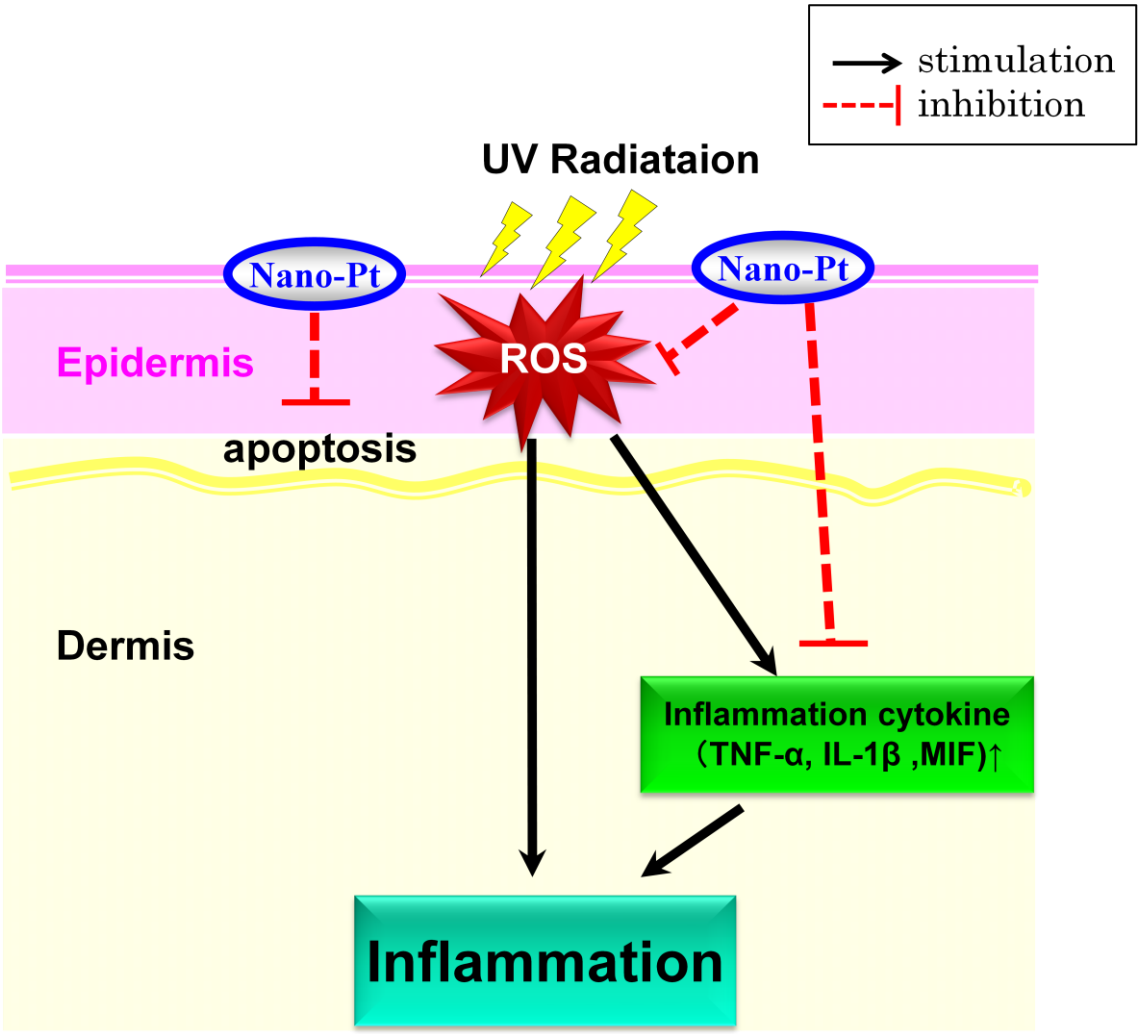


Fig.3 Schematic Diagram of the protective effects of nano-Pt in UV-induced inflammation in the skin [modified from ref. (86)]

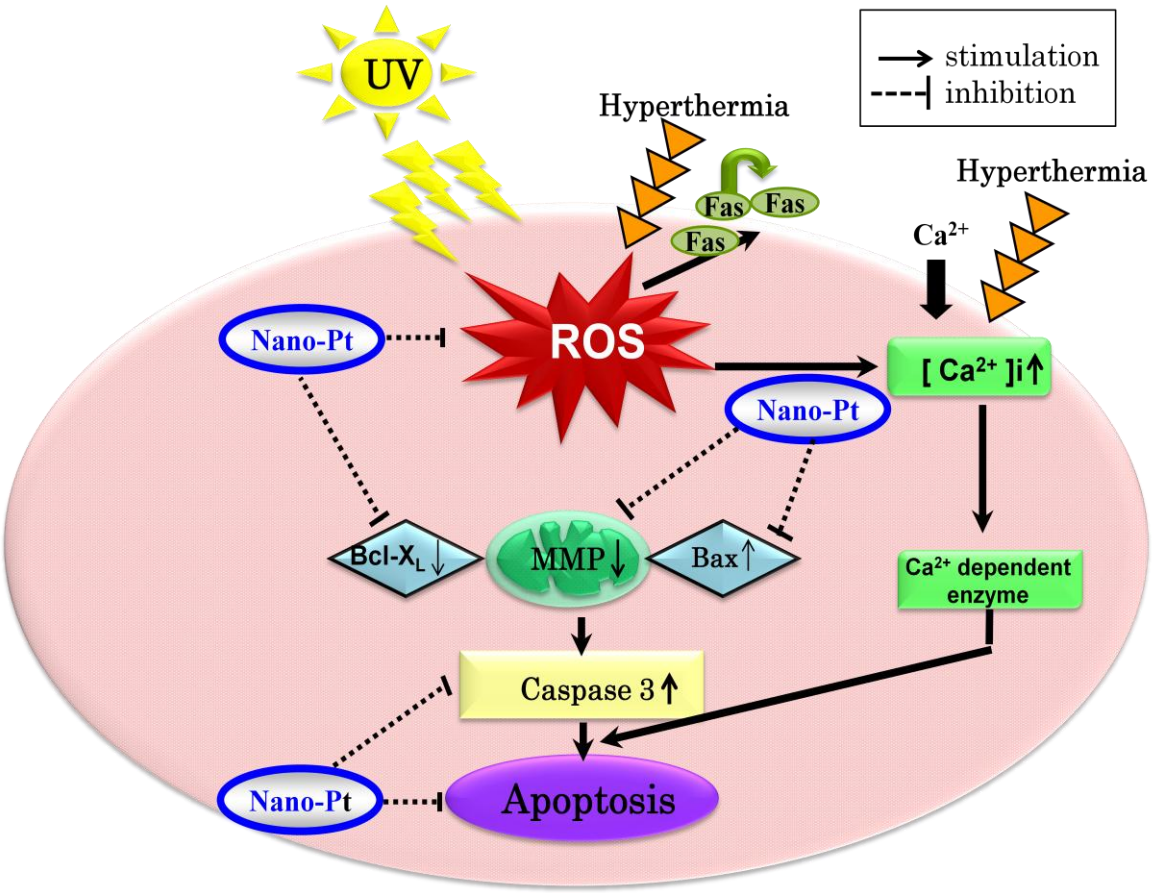


Fig. 4 Schematic view of the suppression of HT- or UV- induced ROS and apoptosis by nano-Pt in cells [modified from ref. (86)]

Chapter 2

The anti-inflammatory effects of platinum nanoparticles on the lipopolysaccharide-induced inflammatory response in RAW 264.7 macrophages

2.1. SUMMARY

Platinum nanoparticles (nano-Pt) have been reported to possess anti-oxidant and anti-tumor activities. However, the biological activity and mechanism of action of nano-Pt in cases of inflammation are still unknown. The present study was designed to determine the *in vitro* anti-inflammatory effects of nano-Pt on lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. RAW 264.7 macrophages were used for the study. The LPS-induced production of reactive oxygen species (ROS) was determined by flow cytometry. The PGE_2 concentration was measured using a PGE_2 assay kit. The protein levels and mRNA expression of the pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), along with cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) were analyzed by a Western blot analysis and RT-PCR. The phosphorylation of extracellular signal regulated kinase (ERK1/2) and Akt, and the phosphorylation and degradation of inhibitory kappa B-alpha ($I_\kappa B$-α) was determined by a Western blot analysis. Nano-Pt significantly reduced the LPS-induced production of intracellular ROS and inflammatory mediators. In addition, nano-Pt suppressed the phosphorylation of ERK1/2 and Akt, and significantly inhibited the phosphorylation/degradation of $I_\kappa B$-α as well as the nuclear factor kappa-B (NF-$_\kappa$B) transcriptional activity. The findings from this study suggest that the anti-inflammatory properties of nano-Pt may be attributed to their downregulation of the NF-$_\kappa$B signaling pathway in macrophages, thus supporting the use of nano-Pt as an anti-inflammatory agent.

2.2. Introduction

Inflammation is one of the most crucial aspects of the host defense against invading pathogens. Aberrant regulation of inflammation has been considered to be one of the major causes of human cancer (9). Factors involved in the inflammatory process include physical and chemical stimulants that are released during the immune response, and by tissue necrosis. Nitric oxide (NO) and reactive oxygen species (ROS) play a significant role in the inflammatory response. During an inflammatory response, the excessive production of ROS can cause major damage to cells, which can lead to DNA damage and mutations (87, 88).

Macrophages are the primary pro-inflammatory cells that provide the first line of defense against these harmful stimuli. In response to extracellular stimuli such as ultraviolet (UV) irradiation or lipopolysaccharide (LPS) stimulation, macrophages mediate the inflammatory response by releasing a variety of pro-inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and prostaglandin E_2 (PGE_2), which exert profound effects on endothelial, epithelial and mesenchymal cells in the local microenvironment (1, 10) .The overproduction of these pro-inflammatory mediators results in aggravated inflammatory responses (89).

Nuclear factor-κB (NF-κB) is one of the key regulators of pro-inflammatory gene

expression. In unstimulated cells, NF-$_\kappa$B is retained in the cytoplasm by binding with a family of inhibitory proteins, the inhibitors of NF-$_\kappa$B (I$_\kappa$B). Upon cell stimulation, the phosphorylation and degradation of I$_\kappa$B-α leads to the translocation of free NF-$_\kappa$B to the nucleus (90, 91). Anomalous activation of NF-$_\kappa$B is a clinical hallmark of chronic inflammation, and is associated with many types of cancer. Compounds having NF-$_\kappa$B inhibitory activity have therefore been found to be useful additions to the chemotherapy regimens for a variety of inflammatory disorders and cancer (92, 93).

Nanotechnology is becoming increasingly important for research on the development of new therapeutic approaches for cancer, inflammation and aging. Nanoparticles are currently being applied not only in material science and engineering, but also in medical science and for clinical use. Nanoparticles can be antioxidants, thus reducing the production of ROS in the body. Polyacrylic acid-protected platinum nanoparticles (nano-Pt) are manufactured by reduction with ethanol for 2 h (80). Platinum-based compounds gained attention after the discovery of the anti-tumor activity of *cis*-diaminedichloroplatinum (83). Recently, we have demonstrated that nano-Pt can exert superoxide dismutase (SOD) catalase mimetic activity, which could be useful in the prevention of a number of oxidative stress-associated pathologies, such as inflammatory reactions and cellular transformation (85).

2.3 Aims of the thesis

The study was aimed to:

- Examine the mechanism of action involved in the anti-inflammatory effects of nano-Pt on RAW 264.7 macrophage cells stimulated by lipopolysaccharide.
- To evaluate the anti-oxidants effects of nano-Pt against lipopolysaccharide induced reactive or nitrogen oxygen species.

Chapter 3

Materials and Methods

3.1. Materials

Nano-Pt were prepared by a method as described previously [80]. The Isogen RNA extraction kit was obtained from Nippon Gene (Tokyo, Japan). M-MLV reverse transcriptase was from GIBCO (Grand Island, NY, USA). *Taq* DNA polymerase was purchased from Perkin-Elmer (Norwalk, CO, USA). The LPS was purchased from Sigma (St. Louis). The anticyclooxygenase-2 (COX-2) polyclonal antibody (pAb) was purchased from Cell Signaling Technology, Inc. (Boston, MA, USA), the anti-inducible nitric oxide synthase (iNOS) pAb was purchased from Enzo Life Sciences International Inc. (NY, USA), and the anti-*β*-actin Ab was from Santa Cruz Biotechnology Inc. (CA, USA). The Western blot detection system was obtained from Cell Signaling Technology (Beverly, MA, USA). All other reagents were of analytical grade.

3.2. Preparation of nano-Pt

Nano-Pt were prepared by the citrate reduction of H_2PtCl_6, according to previous report with minor modifications (80). Briefly, 43.8 ml water was poured into a 100 ml eggplant- type flask and 4 ml of 16.6 mM H_2PtCl_6 was added. The reaction mixture in the flask was stirred at 100 ^oC until reflux started. An 8.6 ml aliquot of 77.2 mM trisodium citrate dihydrate was injected into the reaction mixture and reflux was

continued for additional 30 min. A Change in the color of reaction mixture from light yellow to dark brown or dark red was observed, thus indicating the start of platinum reduction and nanoparticles formation. The reaction mixture was cooled to room temperature, 10 ml of 3.96 mg/ml pectin was added and the mixture was stirred for 1 h. More pectin was added to improve the stability of nano-Pt. Finally, the average diameter of nano-Pt was determined to be 2.4 ± 0.7 nm.

3.3. Cell culture

RAW 264.7 cells (mouse) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cultured cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotics, at 37^{o}C in humidified air with 5% CO_2.

3.4. MTT assay

The Cells were seeded in 96-well plates (2.5×10^3 cells/well) and grown in DMEM for 24 h. Nano-Pt (10, 50, 100, 500 and 1000 μM) were added to cells for 24 h. The, medium was removed, and another medium containing 10% MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution (Sigma, 0.5 mg/ml

in PBS) was added to each well. The cells were incubated for 4 h at 37°C, the supernatants were removed, and dimethyl sulfoxide (Sigma) was added to each well. The optical density was measured at 570 nm using a BIO-RAD microplate reader (Hercules, CA, USA).

3.5. Assessment of intracellular ROS

The intracellular ROS levels were measured flow cytometrically using two different fluorescent probes with different affinities for ROS: hydroethidine (HE) was used to confirm the production of superoxide (O_2^-), while dichlorofluorescein diacetate (DCFH-DA) was used to evaluate the involvement of peroxides, including hydrogen peroxide (H_2O_2). After a 10-min incubation following LPS treatment, cells were stained with 2 μM HE and 5 μM DCFH-DA, and incubated for 30 min at 37^{o}C in the dark. Finally, cell samples were injected into a flow cytometer for the analysis.

3.6. PGE_2 and cytokine assays

RAW 264.7 cells were treated with nano-Pt at various concentrations (10, 50 or 100 μM), and stimulated with LPS (10 μg/ml). The 24-h culture media was collected, and the PGE_2 concentration was measured using a PGE_2 assay kit (R&D Systems,

Minneapolis, MN, USA). The protein and mRNA expression level of IL-1β, IL-6 and TNF-α were determined by a Western blot analysis and reverse transcription chain reaction analysis (RT-PCR).

3.7. Western blot analysis

Cells were collected and washed with cold PBS. The cells were lysed at a density of 1×10^6 cells/50 μL of RIPA buffer (1M Tris-HCA, 5M NaCl, 1% Nonidet P-40 (v/v), 1% sodium deoxycholate, 0.05% SDS, 1mM phenylmethyl sulfonyl fluoride) for 20 min. After brief sonication, the lysates were centrifuged at 12,000 rpm for 10 min at 4^{o}C, and the protein content in the supernatants was measured using a bio-rad protein assay kit (Bio-Rad, Hercles, CA). The protein lysates were denatured at 96^{o}C for 5 min after mixing with 5 μL of sodium dodecylsulfate (SDS) loading buffer, applied on an SDS polyacrylamide gel for electrophoresis, and transferred to nitrocellulose membranes. Western blot analysis was carried out to detect the expression levels of COX-2, iNOS, IL-6, IL-1β, TNF-α, I_κB-α, p- I_κB-α, Akt, p-Akt, ERK1/2, p-ERK1/2, p38, p-p38, SAPK/JNK and p-SAPK/JNK using specific antibodies. Band signals were visualized on X-ray film using chemiluminescence ECL detection reagents (Amersham Biosciences, Buckinghamshire, UK). The relative amounts of proteins associated with

specific antibodies were normalized according to the intensities of *β*-actin. Band density was quantified by a BIO-ID image analyzer, and the relative amounts of proteins associated with specific antibodies were normalized according to the intensities of *β*-actin.

3.8. Reverse transcription-polymerase chain reaction analysis (RT-PCR)

Total RNA was extracted and RNA reverse transcription was performed with M-MLV reverse transcriptase using random hexamer primers, and subsequent amplification was done using *Taq* DNA polymerase. PCR was carried out for 30 cycles with denaturation at 94 ^{o}C for 30 s, annealing from 52 to 64 ^{o}C for 1 min and extension at 72 ^{o}C for 30 s using a thermal cycler (PE Applied Biosystems Gene Amp PCR system 9700). The IL-6 primers used were 5'-GATGCAATAACCACCCCTGACCC-3' (forward) and 5' -CAATCTGAGGTGCCCATGCTAC- 3' (reverse). The TNF-α primers used were 5'-ACACCGTCAGCCGATTTGC-3' (forward) and 5$^{/}$-CCCTGAGCCATAATCCCCTT- 3' (reverse). The iNOS primers used were 5'-TCC TACACCACACCAAAC-3' (forward) and 5'-CTCCAATCTCTGCCTATCC-3' (reverse). The COX-2 primers used were 5' -CTTCACGCATCAGTTTTTCAAG-3'

(forward) and 5' -TCACCGTAAATATGATTTAAGTCCAC-3' (reverse). The IL-1β primers used were 5'-TCAAGGCATAACAGGCTCATC-3' (forward) and 5′-CCACGGGCAAGACATAGGTAG-3' (reverse) (94). GAPDH was used as a positive control. The primers used were 5' -ACCCAGAAGACTGTGGAT-3' (forward) and 5' -TCGTTGAGGGCAATGCCA-3' (reverse). After PCR, the amplified products were analyzed using 2% agarose gel electrophoresis.

3.9. Statistical analysis

Differences between the various treatments were statistically analyzed using Student's *t*-test. For comparisons of multiple groups, one-way ANOVA was applied to the data. $p < 0.05$ was considered statistically significant. Data in the figures are shown as the mean ± SD of several experiments.

Chapter 4

Specific research work

4.1. Cytotoxicity of nano-Pt on RAW 264.7 cells

The cytotoxic effects of nano-Pts (10, 50, 100, 500 and 1000 μM) were evaluated in RAW264.7 cell, which found that nano-Pt exerts no cyototoxic effects on RAW264.7 cells even at a concentration of 1000 μM (Fig.5).

4.2. Nano-Pt inhibit intracellular ROS production in LPS-stimulated RAW 264.7 cells

We first examined the effects of nano-Pt on LPS-induced ROS production in RAW 264.7 cells. The RAW 264.7 cells were treated with LPS at concentrations of 1 and 10 μg/ml for different time intervals, and flow cytometry with HE and DCFH–DA staining was used to detect the ROS production in the cells. A marked increase in the production of O_2^- and H_2O_2 was observed after 10 min of LPS stimulation at the concentration of 10 μg/ml (Fig. 6a). Furthermore, a 24 h pre-incubation with 100 μM nano-Pt significantly inhibited the LPS-induced production of intracellular ROS (Fig. 6b). Accordingly, we used a 10 μg/ml concentration of LPS for the next series of experiments.

4.3. Nano-Pt reduce the protein and mRNA expression levels of iNOS in LPS-stimulated RAW 264.7 cells

To examine the anti-inflammatory activity of nano-Pt, we examined the effects of nano-Pt on the iNOS mRNA and protein expression in the LPS-stimulated macrophages. LPS stimulation of the RAW 264.7 cells strongly upregulated the iNOS protein and mRNA expression levels. As shown in Fig. 7a and b, treatment with nano-Pt (10-100 μM) caused a significant decrease in the protein and mRNA levels of iNOS in a dose-dependent manner. Previously, it was reported that the overproduction of NO is regulated by iNOS, and that it is involved in the pathogenesis of several inflammatory diseases (95). Therefore, on the basis of these results, we speculated that the nano-Pt attenuate the production of NO by reducing the expression of iNOS in LPS-stimulated murine macrophages.

4.4. Nano-Pt reduce PGE_2 production and the levels of COX-2 protein and mRNA expression in RAW 264.7 cells

In order to assess the anti-inflammatory properties of nano-Pt, the inhibitory effects of nano-Pt on the LPS-induced production of PGE_2 were investigated in the RAW 264.7 cells. LPS stimulation resulted in the increased production of PGE_2 by up to tenfold

compared to unstimulated cells, but nano-Pt (10-100 μM) successfully attenuated the increased PGE_2 production in a dose-dependent fashion (Fig. 8a). Furthermore, to investigate a potential mechanism involved in the inhibition of PGE_2, we examined the effects of nano-Pt on the protein and mRNA expression of the COX-2 enzyme. As shown in Fig. 8b, the band density of COX-2 is reduced as compared to *β*-actin, this finding indicate that 100 μM of nano-Pt significantly reduced the protein expression of the COX-2 enzyme. The COX-2 mRNA expression was also significantly decreased in a dose-dependent manner (Fig. 8c). The above findings indicate that the inhibition of PGE_2 occurs via the suppression of COX-2 expression.

4.5. Nano-Pt reduce the release of pro-inflammatory cytokines by LPS-stimulated RAW 264.7 cells

LPS stimulation of RAW 264.7 cells increased their production of pro-inflammatory cytokines, including IL-6, IL-1*β* and TNF-α. After treatment with nano-Pt, these increases were significantly reduced in a dose-dependent manner, as shown in Fig. 9a. These results were further confirmed by comparing the differences in the band densities of these cytokines to *β*-actin using the BIO-ID image analyzer. In addition, nano-Pt also caused a reduction in the LPS-induced mRNA levels of IL-6, IL-*β* and TNF-α (Fig. 9b).

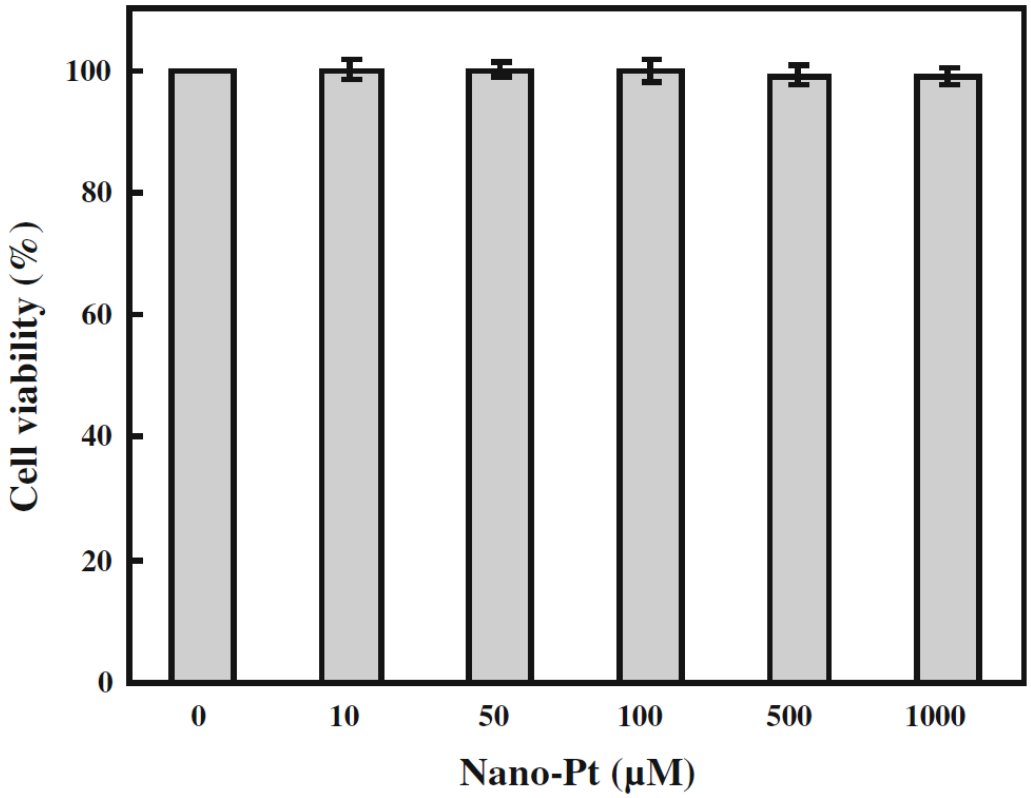
4.6. Nano-Pt inhibit NF-$_\kappa$B signaling and I$_\kappa$B-α degradation in LPS-stimulated RAW 264.7 cells

In the LPS-stimulated RAW264.7 cells, the activation of NF-$_\kappa$B leads to the increased transcription of pro-inflammatory mediators, such as iNOS, COX-2, TNF-α, IL-6 and IL-1β. To investigate the phenomena involved in the inhibition of the NF-$_\kappa$B transcriptional activity, the effects of nano-Pt on the degradation of I$_\kappa$B-α were studied. We observed that nano-Pt blocked the degradation of I$_\kappa$B-α (Fig. 10), suggesting that it significantly attenuates the LPS-stimulated translocation of NF-$_\kappa$B by blocking I$_\kappa$B-α degradation in RAW 264.7 cells.

4.7. Nano-Pt inhibit the phosphorylation of Akt and ERK1/2 in LPS-stimulated RAW 264.7 cells

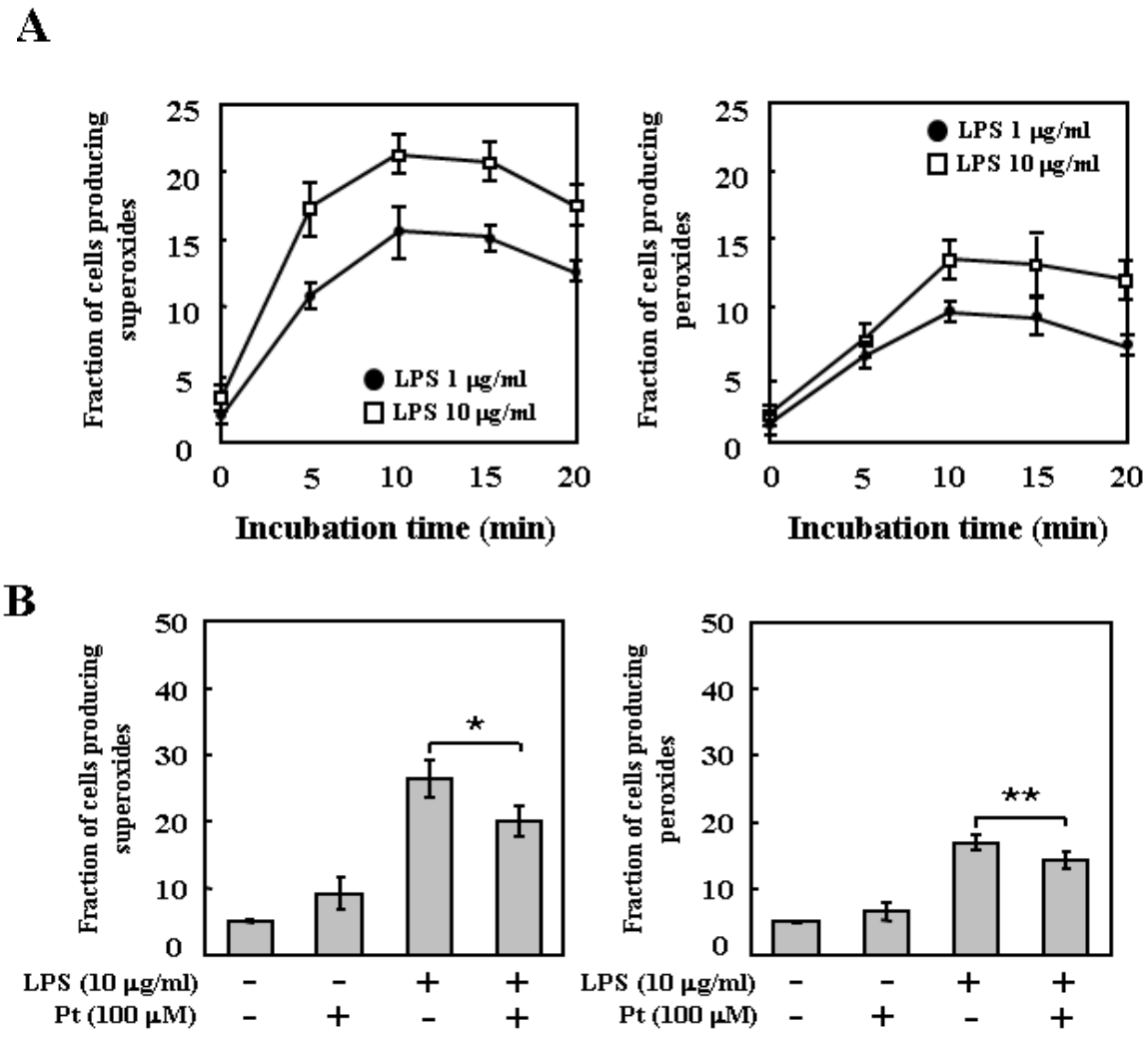
RAW 264.7 cells were stimulated with LPS for 20 min, resulting in the activation of Akt and the MAPK proteins ERK1/2, p38 MAPK, and SAPK/JNK. The treatment of cells with nano-Pt (10-100 μM) for 1 h significantly inhibited the LPS-induced phosphorylation of Akt and ERK1/2. On the other hand, we found that nano-Pt had no inhibitory effect on the phosphorylation of p38 and SAPK/JNK in mouse macrophages (Fig. 11).

Fig. 5 Cell viability of RAW 264.7 cells



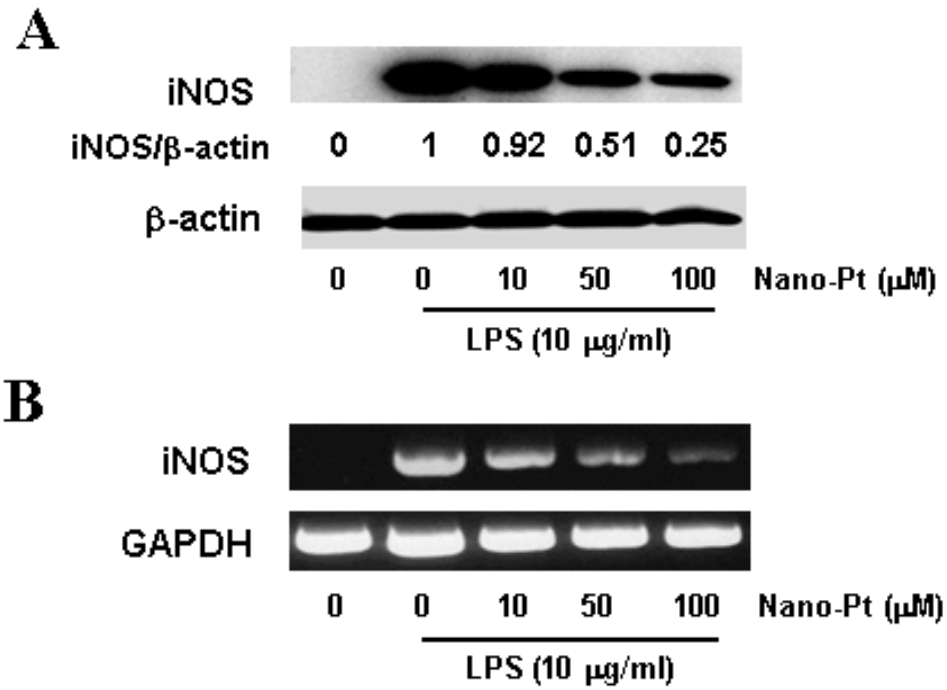
Cell viability of RAW 264.7 cells after incubation in the presence of various concentrations of nano-Pt for 24 h. Cell viability was measured using an MTT assay. Data represent the mean ± SD of three independent experiments.

Fig. 6 The effects of nano-Pt on LPS-induced ROS production in RAW 264.7 cells.



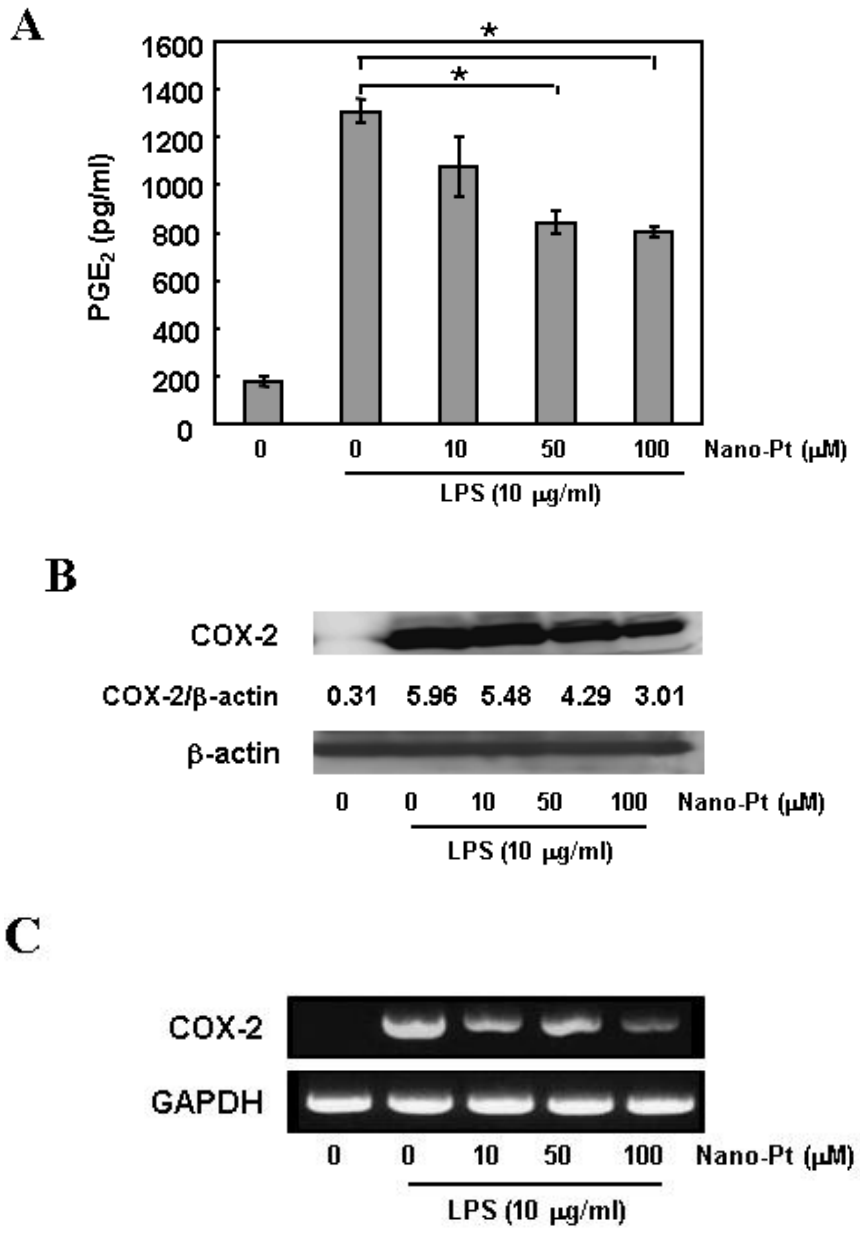
(a) LPS stimulation at a concentration of 1 or 10 μg/ml caused a marked increase in the production of O_2^- and H_2O_2. (b) Cells were pre-incubated for 24 h with 100 μM nano-Pt in the presence and absence of LPS (10 μg/ml) for 10 min. The data are presented as the means±SD (n=5).* $p<0.005$, **$p<0.01$. The data shown are representative of three independent experiments

Fig. 7 The effects of nano-Pt on LPS-induced iNOS protein and mRNA expression in RAW 264.7 cells.



(a) Cells were treated with nano-Pt (10, 50 or 100 μM) for 24 h and then stimulated with LPS (10 μg/ml) for 24 h. Cell lysates were obtained, and the protein levels of iNOS were analyzed via a Western blot with an anti-iNOS or anti-*β*-actin antibody. The iNOS signals were normalized to the *β*-actin signals, and the relative ratios are shown above each band. The anti-*β*-actin antibody was used as an internal control for the Western blot analysis. (b) The total RNA was isolated 6 h after LPS stimulation, and the mRNA levels of iNOS were detected by RT-PCR. Human GAPDH was used as an internal control for the RT-PCR. The data shown are representative of three independent experiments.

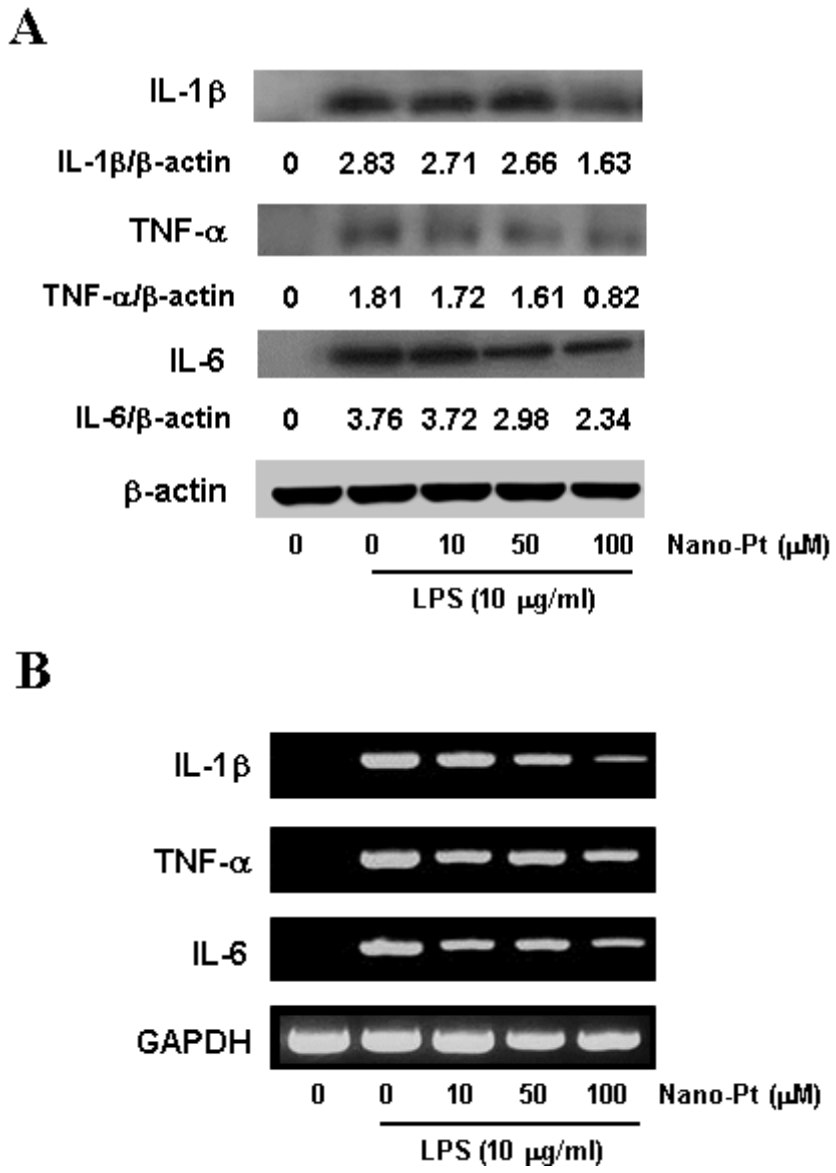
Fig. 8 The effects of nano-Pt on the LPS-induced production of PGE_2 and the expression of COX-2 in RAW 264.7 cells.



Nano-Pt decreased the PGE_2 production and COX-2 expression in LPS-stimulated RAW 264.7 cells. The cells were pretreated with various concentrations of nano-Pt (10,

50 or 100 μM) then stimulated with LPS (10 μg/ml). (a) The 24 hr culture media were collected to measure the PGE_2 concentration. *Each bar* represents the mean ± SD (n=5), *p<0.001. (b) Cell lysates were subjected to a Western blot analysis with an anti-COX-2 antibody or an anti-*β*-actin antibody. *β*-actin was used as an internal control for the Western blot analysis, iNOS signals were normalized to the *β*-actin signals, and the relative ratios are shown above each band (c) The total RNA was isolated, and the mRNA expression of COX-2 in cells was detected by RT-PCR, using human GADPH as an internal control for the RT-PCR. Representative results from three independent experiments are shown

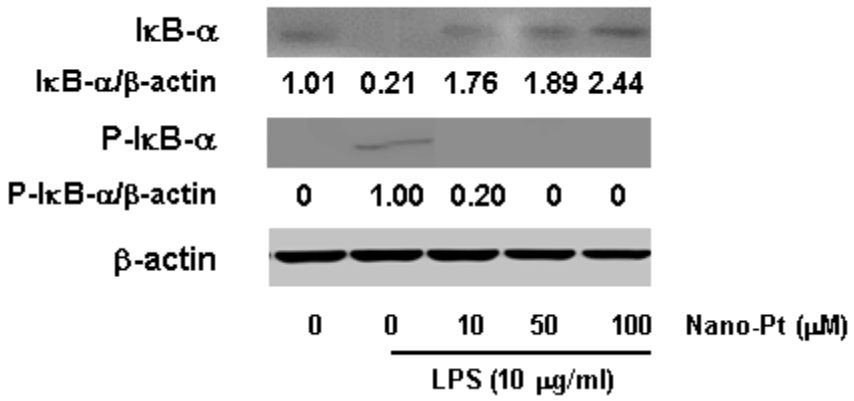
Fig. 9 The effects of nano-Pt on the LPS-induced expression of pro-inflammatory mediators in RAW 264.7 cells.



The cells were pretreated with various concentrations of nano-Pt (10, 50 or 100 μM) and stimulated with LPS (10 μg/ml). (a) Cell lysates were extracted, and the protein expression levels of TNF-α, IL-6 and IL-1β were measured by a Western blot analysis.

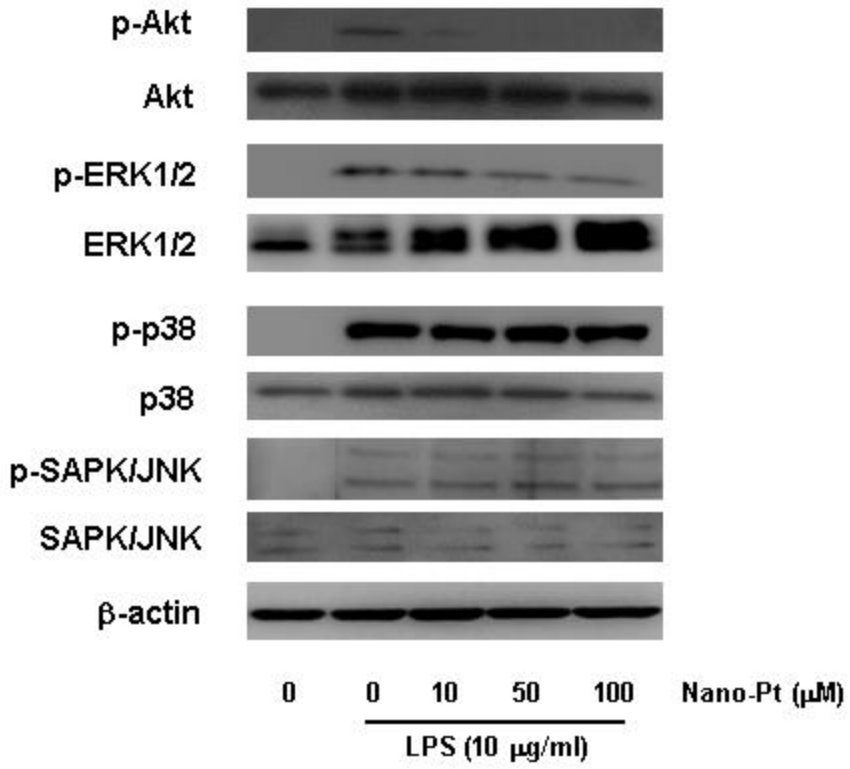
The β-actin expression was used as an internal control for the Western blot analysis, the relative ratios are shown above each band (b) Total RNA was prepared by RT-PCR, and the mRNA expression levels of TNF-α, IL-6 and IL-1β were detected, using human GADPH as an internal control for the RT-PCR

Fig. 10 The effects of nano-Pt on the LPS-induced phosphorylation/degradation of $I_{\kappa}B$-α in RAW264.7 cells.



The RAW264.7 cells were treated with different amounts of nano-Pt (10, 50 or 100 μM) for 24 h, and then stimulated with LPS for 20 min. The cell lysates were extracted, and subjected to Western blot analysis using anti- $I_{\kappa}B$-α antibodies. *β*-actin was used as an internal control. These results are representative of three independent experiments

Fig. 11 The effects of nano-Pt on the LPS-induced phosphorylation of mitogen-activated protein kinases in RAW 264.7 cells.



Nano-Pt significantly inhibited the phosphorylation of Akt and ERK1/2 induced by LPS in RAW 264.7 cells. The cells were treated with nano-Pt for 1h, then stimulated with LPS and incubated for 20 min. To determine the protein expression levels, the cell lysates were analyzed by a Western blot analysis

Chapter 5

Discussion and Conclusion

5.1. Discussion

In this study, we demonstrated that LPS stimulation of RAW264.7 cells enhanced their accumulation of intracellular ROS. NO and ROS are known to be crucial inflammatory mediators. ROS accumulation is an important factor in the pathogenesis of many diseases, including inflammation and cancer (96). ROS are also considered to be implicated in the expression of inflammatory genes via the redox-based activation of NF-κB signaling pathway (97). Pretreatment of cells with nano-Pt significantly reduced the LPS-induced ROS production. We previously reported that nano-Pt effectively protects against UV-induced inflammation by decreasing ROS production (84). Nano-Pt are known to possess strong antioxidant activity and are capable of scavenging ROS (80**)**. A recent study also demonstrated that nano-Pt significantly inhibited RANKL-stimulated osteoclastogenesis in RAW 264.7 cells by the suppression of ROS (98). Overproduction of NO is regulated by iNOS, and is involved in the pathogenesis of several inflammatory diseases (95). In the present study, Nano-Pt were also found to reduce the expression of iNOS mRNA and protein, demonstrating that nano-Pt effectively inhibits NO production via the downregulation of iNOS expression.

Cisplatin is a platinum-based type of chemotherapy and is one of the cytotoxic agents commonly used in cancer chemotherapy. Cisplatin exerts its activity by

interfering with transcription and other DNA-mediated cellular functions (99). Recently, it was reported that nano-Pt have anti-tumor activity, affecting DNA integrity (100). The anti-tumor activity of nano-Pt may be due to the lethal DNA damage effects, caused by soluble Pt ion species, resulting in DNA strand breaks and formation of Pt-DNA complexes (101). In contrast, the inhibitory effects of nano-Pt on LPS induced ROS were observed in the current study. Our results are consistent with the previous report, which showed that various size of nano-Pt (<20, <100, >100 nm) exert no influence on proliferation or growth inhibitory effects (101), indicating that nano-Pt do not exhibit any serious cytotoxity.

Nano-Pt reduced PGE_2 production induced by LPS in a dose-dependent manner. The Western blot analysis and RT-PCR assay showed that nano-Pt also inhibits the protein and mRNA expression of COX-2. These results indicate that nano-Pt significantly attenuated PGE_2 production via the inhibition of COX-2 mRNA and protein, thus suggesting that the specific inhibition of iNOS and COX-2 might be responsible for the anti-inflammatory activity of nano-Pt. Activated macrophages release pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α. TNF-α promotes the induction of IL-6 and IL-1β; high levels of these cytokines can enhance malignancy and cause systemic complications (1, 102). Nano-Pt suppressed the release of TNF-α, IL-6 and IL-1β in

LPS-stimulated macrophages, showing that nano-Pt can attenuate the production of pro-inflammatory cytokines.

We also found that nano-Pt inhibited the LPS-induced phosphorylation of ERK1/2 and the phosphorylation/degradation of $I_{\kappa}B$-α in a dose-dependent manner, which suggested that nano-Pt can inhibit NF-$_{\kappa}$B activation by hampering the phosphorylation of ERK1/2 and subsequent phosphorylation/degradation of $I_{\kappa}B$-α in LPS-stimulated RAW264.7 cells. Furthermore, LPS stimulation of mouse macrophages induces the phosphorylation of Akt; previous studies have shown that the activation of the Akt signaling pathway enhances the degradation of $I_{\kappa}B$-α and leads to the activation of NF-$_{\kappa}$B (103). The LPS-induced phosphorylation of Akt was also inhibited by nano-Pt.

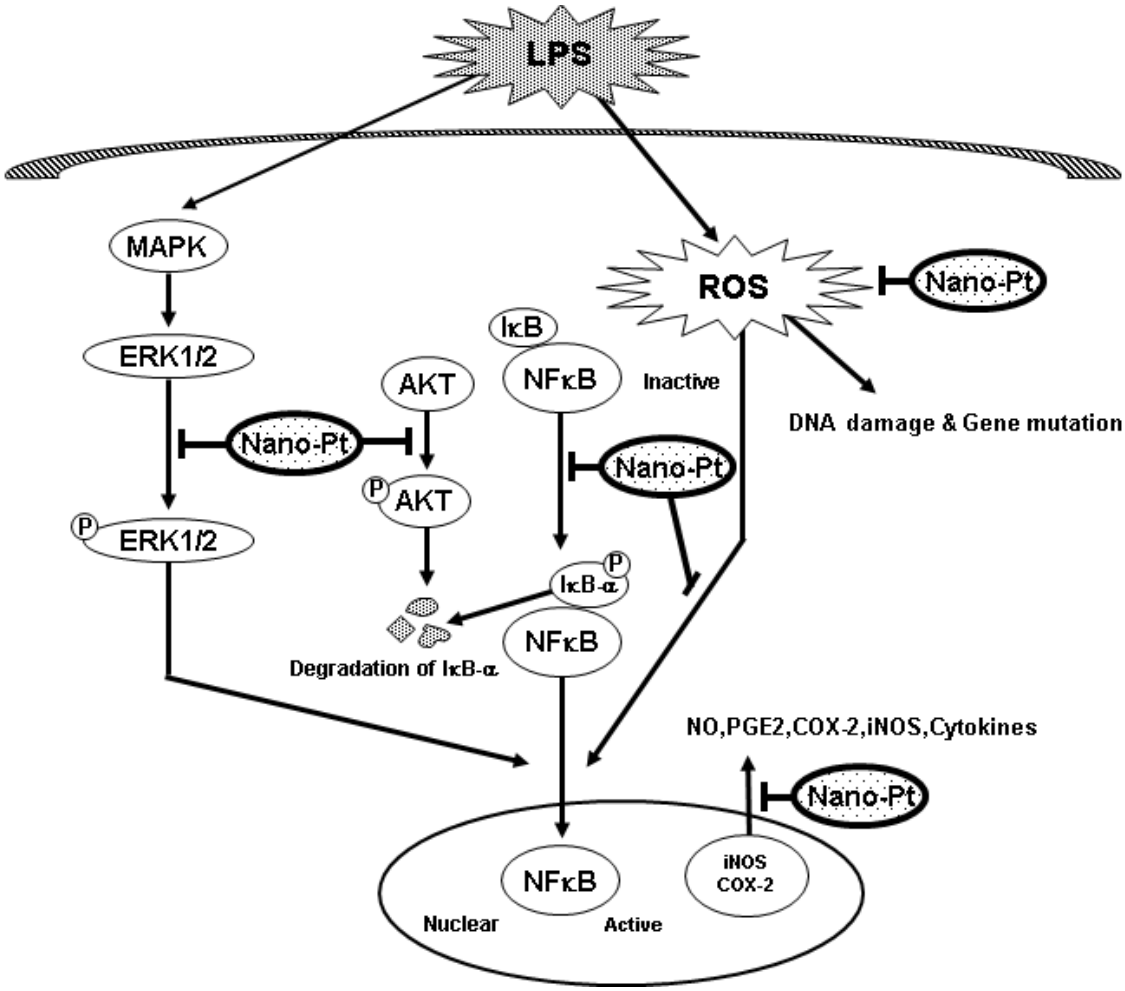
In our search for the mechanism(s) underlying the anti-inflammatory effects of nano-Pt, we focused on two distinct signaling pathways, the NF-$_{\kappa}$B and MAPK pathways. In response to extracellular stimuli, $MAPK_S$ can be activated and can cause consecutive phosphorylation of several important signaling molecules that regulate cell growth, inflammation and apoptosis (104, 105). Several previous reports have documented that the specific MAPK inhibitors also inhibit NF-$_{\kappa}$B transcriptional activity, demonstrating that MAPKs are crucial factors for the activation of NF-$_{\kappa}$B (106, 107). Activated NF-$_{\kappa}$B acts as a transcription factor, leading to increased expression of

several inflammatory genes, such as iNOS, TNF-α, COX-2, IL-1β and IL-6 (108). Taken together, our findings revealed that the anti-inflammatory effects of nano-Pt might be attributable to the suppression of Akt and ERK activation and the subsequent inhibition of NF-$_\kappa$B, thus resulting in the reduced expression of iNOS, COX-2, and inhibition of ROS and inflammatory cytokines.

5.2. Conclusion

In conclusion, this study provides a description of at least one molecular mechanism involved in the anti-inflammatory effects of nano-Pt on macrophages. The anti-inflammatory effects of nano-Pt were attributed to the suppression of Akt and ERK phosphorylation and the subsequent inhibition of NF-$_\kappa$B (Fig. 12), suggesting that nano-Pt could be used as an effective anti-inflammatory agent.

Fig. 12 Graphical scheme of anti-inflammatory effects of nano-Pt



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