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Review article

Retinoic acid and cancer treatment

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ABSTRACT

Retinoic acid which belongs to the retinoid class of chemical compounds is an important metabolite of vitamin A in diets. It is currently understood that retinoic acid plays important roles in cell development and differentiation as well as cancer treatment. Lung, prostate, breast, ovarian, bladder, oral, and skin cancers have been demonstrated to be suppressed by retinoic acid. Our results also show that low doses and high doses of retinoic acid may respectively cause cell cycle arrest and apoptosis of cancer cells. Also, the common cell cycle inhibiting protein, p27, and the new cell cycle regulator, Cdk5, are involved in retinoic acid's effects. These results provide new evidence indicating that the molecular mechanisms of/in retinoic acid may control cancer cells' fates. Since high doses of retinoic acid may lead to cytotoxicity, it is probably best utilized as a potential supplement in one's daily diet to prevent or suppress cancer progression. In this review, we have collected numerous references demonstrating the findings of retinoic acid in melanoma, hepatoma, lung cancer, breast cancer, and prostate cancer. We hope these observations will shed light on the future investigation of retinoic acid in cancer prevention and therapy.

1. Introduction

Vitamins are nutrients essential for the body's growth, differentiation, development, and protection., Vitamin A is especially important because it can't be synthesized by animals and must be supplied from a diet that includes plants [1]. There are many derivatives of vitamin A, including β-carotene, retinol, retinal, isotetrinoin, and retinoic acid. Treatment using retinoic acid was approved by the U.S. Food and Drug Administration for lymphoma [2] and leukemia [3]. Since retinoic acid is known to be effective in treating cancer, its basic structure has been well identified. All of the retinoids, including retinoic acid, are comprised of three units: a bulky hydrophobic region, a linker region, and a polar region (carboxylic acid terminus). There are many compounds derived from the above basic structure, and these compounds are collectively called retinoids [1]. Due to the efficiency of natural retinoids in cancer treatment, synthetic retinoids have been generated and investigated. In anti-cancer research, retinoic acid has been investigated and found to inhibit the markers of cell proliferation, such as cyclin D1 and human telomerase reverse transcriptase (hTERT), and growth factor, such as epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) [1]. The biological functions inhibited by retinoic acid include tumor growth, angiogenesis, and metastasis [1]. In addition, retinoic acid has also been found to regulate mitochondrial permeability, death receptors, ubiquitination, and reactive oxygen species, etc. [4]. It is believed that the inhibitory effects of retinoic acid are achieved through activating the retinoic acid receptor (RAR) or retinoic X receptor (RXR). RAR and RXR form heterodimers and function after ligand binding. To turn on downstream gene expression, RAR and RXR shuttle into cell nuclei and bind to the retinoic acid response elements (RARE), which are located in the 5'-region of retinoic acid downstream genes [5]. The activation of the above classical pathway will lead to cell differentiation, arrest, and eventually apoptosis [6]. In addition to the above classic pathway, retinoic acid may also regulate the downstream gene expression through modulating other transcription factors, such as NF-κB, IFN-γ, TGF-β, MAPK, and even chromatin remodeling [4]. RARs/RXRs heterodimerize with other receptors and regulate these partner receptors' signaling, including non-classical or non-genomic pathways [7]. Sometimes, these partner receptors have opposite functions to RARs/RXRs. The latest finding of retinoic acid is the regulation of stem cell differentiation [8]. Ying *et al*. found that retinoic acid induces the expression of lineage-specific differentiation markers Tuj1 and GFAP and reduces the expression of neural stem cell markers such as CD133,

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Msi-1, nestin, and Sox-2 [8]. In their expression microarray analysis, retinoic acid-affected pathways include retinoid signaling and metabolism, cell adhesion, cell-matrix interaction and cytoskeleton remodeling. Notch pathway down-regulation was also reported by retinoic acid-induced HES and HEY inhibition [8].

Although there are several lines of evidence indicating the effects and mechanisms of retinoic acid in cancer therapy, the chemo-prevention and therapeutic application of retinoic acid remain controversial. Here, this mini-review article demonstrates an overview of the research to date in the field of retinoic acid application and therapy to various types of cancer. The hope is that this review may impart readers with a better understanding of the research history of retinoic acid as well as guide the future direction in the field.

2. Retinoic acid and melanoma

Retinoic acid has been found to have inhibitory effects on growth of murine melanomas [9] and colony formation of human melanomas [10]. Activations of cyclic AMP-dependent protein kinase and sialyltransferase have also been found to involve the effects of retinoic acid [7, 11]. On the other hand, the modulation of melanoma cell adhesion to basement membrane components has been shown to be affected by retinoic acid treatment [12, 13]. Intercellular adhesion molecule gene I (ICAM-1) is transcriptionally regulated by retinoic acid in melanoma cells [14]. Retinoic acid has also been indicated to inhibit highly metastatic B16F10 melanoma cells by down-regulating the cell surface integrin receptors against extracellular matrix proteins, specifically laminin and vitronectin [15]. Since the formation of melanoma is correlated to radiation, retinoic acid has been found to modify the radio-sensitivity and recovery from X-ray damage *in vitro* [16]. Notably, the induction of protein kinase C in mouse melanoma cells was identified by retinoic acid treatment [17]. Ultraviolet irradiation may deplete cellular retinol and alter the metabolism of retinoic acid in cultured human keratinocytes and melanocytes [18]. In addition to inhibiting growth, retinoic acid has been found to inhibit human melanoma tumor cell invasion [19]. Epidermal growth factor receptor (EGFR) is a crucial player in epithelial cells in both growth and migration/invasion. Yongshan *et al*. discovered that EGFR expression was regulated by retinoic acid treatment [20]. In 1993, the combination treatment of interferon-α and retinoic acid was first believed to have significant therapeutic effect on melanoma by clinical examination [21]. The antitumor effect of green tea polyphenol on melanoma was enhanced by retinoic acid [22]. Interestingly, the differential regulation of tyrosinase activity in the skin of white and black individuals *in vivo* by retinoic acid was demonstrated [23]. In regards to drug delivery improvement, retinoic acid was encapsulated by liposome to treat melanoma cells and was then implanted onto C57BL/6 mice, with result of metastatic ability being efficiently suppressed [24]. A hyaluronic acid-based multifunctional nano-carrier was also used to deliver retinoic acid in cancer treatment tests [25]. All things considered, Retinoic acid seems to be a promising treatment for melanoma and more details will be investigated in the future to strengthen the basis of its mechanism.

3. Retinoic acid and hepatoma

Hepatoma is a serious form of cancer in Asia. It has been found that retinoic acid may directly cause the increase in protein synthesis of transferrin and albumin in Hep3B cells [26]. Since hepatitis virus infection is important to hepatoma formation, Hsu *et al*. demonstrate that retinoic acid may regulate the gene expression of hepatitis B virus surface antigen (HBsAg) in hepatoma cells [27]. Much cancer research focuses on the involvement of topoisomerase in cancer cell growth. Tsao *et al*. has reported that retinoic acid represses the expression of topoisomerase II in Hep3B cells [28]. The most current research of retinoic acid has used the model of short-term treatment and therefore been questioned in clinical therapy. However, Hsu *et al*. have demonstrated that long-term treatment with retinoic acid (30 days) may lead to suppression of the tumorigenicity of human hepatoma cells [29]. Furthermore, apoptosis of hepatoma cells was found after retinoic acid treatment and prevented by serum albumin and enhanced by lipoidol [30]. In addition, p21 induction and cdc2 activation are found to involve retinoic acid-induced hepatoma apoptosis [31]. Since retinoic acid may cause detachment of cancer cells under serum starvation, proteolysis of integrin α5 and β1 subunits were found in hepatoma cells [32]. The latest research indicates that retinoic acid may cooperate with arsenic to induce apoptosis and modulate the intracellular concentration of calcium in hepatoma cells [33]. Additionally, the retinoic acid receptor-related receptor α is believed to be a prognostic marker for hepatoma [34]. Taken together, these observations elucidate the fact that retinoic acid is indeed a potential compound to suppress hepatoma growth and cause hepatoma apoptosis. It's also possible that retinoic acid can work as a helper that cooperates with other treatments and attacks hepatoma.

4. Retinoic acid and lung cancer

The incidence and mortality rates of lung cancer make this disease an important topic in cancer research. Since the relevant contribution of retinoic acid in cancers was discovered, there have been numerous studies demonstrating the effects of retinoic acid in lung cancer progression. At first, Hsu *et al*. found retinoic acid-mediated G1 arrest to be associated with induction of p27 and Cdk3 inhibition in lung squamous carcinoma cells [35]. In C57BL/6 mice model, retinoic acid was encapsulated and inhibited lung cancer metastasis [36]. Syndecan-1 is a proteoglycan that mediates cell-cell adhesion and prevents invasion in epithelial cells. Retinoic acid may increase syndecan-1 expression to block invasion/metastasis of lung cancer [37]. Notably, retinoic acid has been found to reduce chemotherapy-induced neuropathy in an animal model as well as patients with lung cancer [38]. These results show the relevance of retinoic acid in lung cancer treatment.

5. Retinoic acid and breast cancer

The application of retinoic acid in breast cancer treatment was first mentioned in 1970's [39]. A retinoic acid-binding protein is believed to be an important factor in the progression of breast cancer [40, 41]. The latest report indicates that the sensitivity of retinoic acid in triple negative breast cancer cell lines may be restored by other treatment, such as curcumin [42]. Aldehyde dehydrogenase 1A3 (ALDH1A3) influences breast cancer progression *via* differential retinoic acid signaling [43]. Besides the above, a different type of protein kinase C was also found to

involve the induction of the retinoic acid system in breast cancer [44]. Notably, retinoic acid may induce re-differentiation of early transformed breast epithelial cells [45], suggesting the preventive role retinoic acid plays with respect to breast cancer. Kamal *et al*. drew attention to the effect of retinoic acid by proteomic analysis in breast cancer cell lines [46]. The amplification of the retinoic acid receptor α (RARα) and retinoic acid sensitivity were found to correlate to breast cancer progression [47]. Retinoic acid can impair estrogen signaling in breast cancer cells by interfering with the activation of LSD1 via protein kinase A [48]. Retinoic acid was also found to reduce breast cancer growth and lung metastasis [49]. The procoagulant activity of breast cancer cells was reported to be modulated by retinoic acid [50]. Interestingly, microRNA-21 was found to be induced by retinoic acid in breast cancer, which suggests the biological correlation and molecular targets in breast cancer [51]. In addition, retinoic acid may inhibit aromatase activation and expression, which indicates that the estrogen supply inside breast cancer cells is insufficient to maintain cancer cell growth [52]. In addition to growth inhibition, retinoic acid is able to down-regulate MMP-9 by modulating its regulatory molecules and therefore impacts the invasion ability of breast cancer cells [53]. Additionally, retinoic acid may inhibit telomerase activation through inducing histone deacetylation in estrogen receptor-negative breast cancer cells [54]. Importantly, Hau *et al*. elucidated the genomic antagonism between retinoic acid and estrogen signaling in breast cancer and published their findings in the journal, Cell [55]. Their article shows the critical and solid thought of retinoic acid application to breast cancer. Since HOXA5 plays a role in apoptosis of breast cancer cells, retinoic acid was reported to regulate HOXA5 through RAR-β [56]. Cell cycle control gene, Btg2, is believed to be a direct target for RAR signaling in breast cancer cells [57]. Moreover, retinoic acid may sensitize breast cancer cells to taxol through down-regulation of survivin and promote the aberrant mitotic progression that causes apoptosis [58]. Although a lot of evidence demonstrates the effectiveness of the application of retinoic acid to breast cancer, combination treatments with other effective compounds (such as tomaxifen, taxol, and interferone) has been proposed and is currently utilized.

6. Retinoic acid and prostate cancer

Just like breast cancer, the history of retinoic acid treatment for prostate cancer has a strong history going back to the 1980's. Researchers' attention then was focused on the retinoic acid receptor in the study of prostate cancer cells [59]. The effects of retinoic acid on the growth and morphology of a prostate cancer cell line was first investigated [60]. After that, the binding proteins of retinoic acid were identified [61-63]. Since prostate cancer cells are eager to require androgen supplement in the early stages of the disease, 5α-reductase becomes important to provide potent androgens. Retinoic acid was found to inhibit 5α-reductase and therefore became a possible treatment for prostate cancer [64, 65]. Notably, the relationship between retinoic acid and prostate cancer growth was officially mentioned by Whelan [66]. Fong *et al*. demonstrated that retinoic acid at 10 μM may cause inhibition of androgen-dependent prostate cancer cell growth but may cause stimulation when the concentration is 0.01 μM [67]. The growth of androgen-independent prostate cancer cells is also suppressed by retinoic acid [68]. Extracellular matrices were also found to be regulated by retinoic acid [69, 70]. Specifically, retinoic acid has been found to activate the tumor suppressor, Rb, and decline androgen receptor proteins, thereby causing apoptosis of prostate cancer cells [71]. Interestingly, retinoic acid has been reported to interact with androgen in prostate cancer cells, which affects cell proliferation and expressions of retinoic acid receptor and epidermal growth factor receptor [72]. There is some research that demonstrates that the retinoid X receptor (RXR) might play important roles in tumorigenesis of prostate [73, 74]. RXR was also found to involve retinoic acid-induced inhibition of androgen receptor [75]. Hypermethylation of the retinoid acid receptor β is believed to be a prognostic marker in prostate cancer [76, 77]. Notably, the retinoic acid synthesis gene aldehyde dehydrogenase, ALDH1A2, is believed to be a candidate tumor suppressor in prostate cancer [78], which is similar to breast cancer as described above. More solid evidence has been provided by Huss *et al*., in which they have indicated that retinoic acid may slow the progression of prostate cancer and promote apoptosis of cancer cells [79]. In addition, retinoic acid was found to regulate the formation and degradation of gap junctions in prostate cancer cells [80]. Also, retinoic acid may inhibit the proliferation of prostate cancer cells through reducing the methylation level of the HOXB13 gene [81] and the Cdk5-dependent p27 expression [82]. Instead of growth inhibition, high doses of retinoic acid may cause apoptosis of prostate cancer cells though p35 cleavage and Cdk5 overactivation [83]. Although clinical trials have not shown strong evidence indicating that retinoic acid is an effective drug for prostate cancer [84, 85], more and more effort has been put toward retinoic acid research as it relates the nutritional supply and combination therapies with respect to prostate cancer.

7. Conclusion

Retinoic acid has been investigated extensively for its use in treating different forms of cancer not only in prevention but also in treatment. In this review, we described the research and applications of retinoic acid in melanoma, hepatoma, lung cancer, breast cancer, and prostate cancer. As a nutrient, retinoic acid may be obtained from either through the daily metabolization of plants in a balanced diet or through vitamin supplements. Under normal circumstances in the body, retinoic acid does preventive work against cancer formation. After cancer formation, retinoic acid becomes an attacker to cancer cells, one that blocks their growth and division and also triggers their differentiation and death through specific pathways. Furthermore, retinoic acid has been proven to cooperate with other effective cancer therapeutic drugs against cancer progression. Retinoic acid becomes a helper to chemo-therapeutic agents, a helper which may decrease both the dosages of these chemo-therapeutic agents required and their side-effects. This may relieve patients' pain from chemotherapy and improve patients' quality of life. From these points of view, although there has been a long history and no small amount of controversy regarding retinoic acid application in cancer treatment, it's still worthwhile to continue research and place future effort toward gaining a more complete understanding of the application of retinoic acid in cancer treatment.

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Review article

Molecular targets for anti-oxidative protection of green tea polyphenols against myocardial ischemic injury

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ABSTRACT

Ischemic heart disease is the leading cause of death worldwide. An improved understanding of the mechanisms involved in myocardial injury would allow intervention downstream in the pathway where certain drugs including natural products could be efficiently applied to target the end effectors of the cell death pathway. Green tea polyphenols (GTPs) have potent anti-oxidative capabilities, which may account for their beneficial effects in preventing oxidative stress associated with ischemia injury. Although studies have provided convincing evidence to support the protective effects of GTPs in cardiovascular system, the potential end effectors that mediate cardiac protection are only beginning to be addressed. Proteomics analyses widely used to identify the protein targets for many cardiovascular diseases have advanced the discovery of the signaling mechanism for GTPs-mediated cardio-protection. This review focuses on putative triggers, mediators, and end effectors for the GTPs-mediated cardio-protection signaling pathways engaged in myocardial ischemia crisis, allowing a promising natural product to be used for ameliorating oxidative stress associated with ischemic heart diseases.

1. Introduction

Green tea polyphenols (GTPs) have attracted much interest in prevention of atherosclerosis and cardiovascular diseases [1-7]. Epidemiological studies have established a close correlation between the consumption of green tea and protection against cardiovascular diseases and risk factors [8-12]. Other experimental studies on myocardial ischemia injury have also suggested that the cardio-protective effect of GTPs is associated with the scavenging of active-oxygen radicals, the modulation of redox-sensitive transcription factors (e.g., NFκB, AP-1), the reduction of STAT-1 activation and Fas receptor expression, an increase in NO production, the exertion of positive inotropic effects, and the modulation of myofilament Ca^{2+} sensitivity [13-21]. However, limited information is known for the potential end effectors in the GTPs-conducted signaling pathways for cardiac protection. This review intends to increase our understanding on the GTPs-mediated cardio-protective mechanism by which molecular targeting for their anti-oxidative interventions on myocardial ischemic disorder is discussed.

2. Anti-oxidative capacities of GTPs

Oxidative stress describing an imbalance between the generation and clearance of reactive oxygen species (ROS) in cells has been associated with hypoxia or myocardial ischemia, and likely contributes to the progression of cardiovascular diseases [22]. Accumulating evidence also indicates that redox-sensitive signaling pathways *via* the effects of generation of ROS or reactive nitrogen species (RNS) or reactive lipid derived aldehydes (LDAs) are essentially involved in the pathological stress of heart cells [23]. Accordingly, molecular targeting for anti-oxidative interventions on redox signaling pathways may provide a therapeutic approach to ameliorate the risk and progression for heart diseases.

GTPs have potent antioxidant and radical-scavenging properties, which may partially account for their cardio-protective effects [24]. *In vitro*, they have been shown to scavenge ROS or RNS, chelate metal ions, prevent the activation of redox-sensitive transcription factors, inhibit ROS generating enzymes, and increase antioxidant enzymes [25, 26]. The major catechins in GTPs include epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epigallocatechin-3-gallate (EGCG) [27, 28]. These compounds (i.e. biologically active polyphenolic flavonoids) contain two or more aromatic rings, each bearing at least one aromatic hydroxyl connected with a carbon bridge. EGCG is the most physiologically potent compound, and primarily accounts for the biological effects of green tea [2]. Studies with a cell line of H9c2 rat cardiomyoblasts associated with H_2O_2-induced oxidative stress also demonstrated the protective role of

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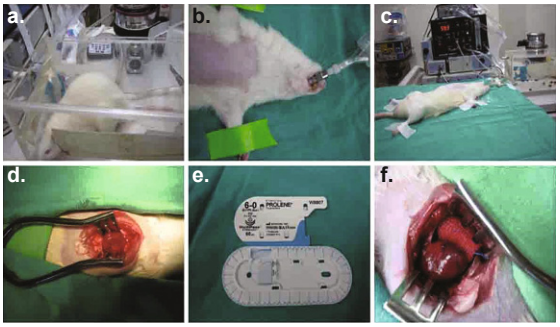
MI surgical model



IR surgical model:
20 min ischemia (I) followed by reperfusion (R)

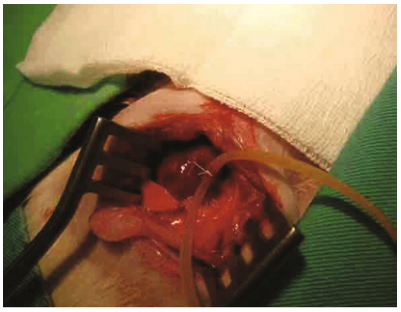


Fig. 1 - Myocardial ischemia models of chronic myocardial infarction (MI) and transient ischemia-reperfusion (IR) created in rats by ligating the left anterior descending coronary (LAD).

EGCG against oxidative injury and cell death caused by ROS and cytosolic Ca^{2+} overload in cardiac cells [29-31].

3. GTPs mediated cardiac protection against myocardial ischemic injury

3.1. Animal models for cardiac adaptation to oxidative stress and myocardial ischemia

Two different myocardial ischemia models (Figure 1) associated with chronic myocardial infarction (MI) and transient ischemia-reperfusion (IR) were created in rats by ligating the left anterior descending coronary (LAD) for studying myocardial ischemic injury [13, 14]. In the MI model, severe myocardial infarction was found in post-MI rats [14], while the IR model involving brief regional ischemia for 20 min followed by subsequent reperfusion showed no severe infarcted injury [13]. These findings suggested that brief regional ischemia followed reperfusion may lead to activate pathways that either preserve cell viability (preconditioning) or lead to cell death (IR injury). In contrast, irreversible MI caused by death of myocytes, presumably as a result of both necrosis and apoptosis, mostly appears within the infarct and peri-infarct regions [32-35].

Reperfusion injury of ischemic tissue is known to be accompanied by the production of ROS and Ca^{2+} overload in injured cardiomyocytes [36-43]. The rise in cytosolic Ca^{2+} levels could induce mitochondrial Ca^{2+} ($[Ca^{2+}]m$) accumulation *via* the mitochondrial Ca^{2+} uniporter and the increased ROS production. Both the $[Ca^{2+}]m$ overload and increased ROS generation would induce opening of the mitochondrial permeability transition pore (mPTP) and rupture of the plasma membrane, triggering cell death [44, 45].

3.2. Pretreatment of GTPs protects myocardial ischemia injury in post-IR rats

A previous study by Miwa *et al.* using isolated hearts perfused with a Langendorff's apparatus showed that GTPs pre-treatment (1 mM, 35 ml/day for 14 days), administered orally prior to surgery, could protect hearts from oxidative stress after reperfusion and avoid cell edema [46]. This result suggested that GTPs might be used as a novel method for preparative cardiac surgery in the future [46]. In addition, other study using a post-IR model in rats also demonstrated that GTPs pretreatment for 4 hours prior to IR injury protects cardiomyocytes by preventing cytosolic Ca^{2+} overload, myofibril disruption, and alterations in adherens and gap junction protein expression and distribution [13].

3.3. GTPs attenuate myocardial remodeling injury in post-MI rats

Myocardial infarction (MI) largely resulting from cardiac ischemic injury often undergoes to cardiac remodeling process, which may cause secondary damage to the heart tissue by excessive ROS and free radicals [47]. A previous study with post MI rat model showed that GTPs reduced heart tissue remodeling injury, avoided ventricular hypertrophy, reduced infarct size, as well as significantly improved the left ventricular functions [14]. Using the same post MI model, the rate of intracellular free radicals produced in cardiomyocytes extracted from post MI rats with GTPs treatment for 3 days, 2 weeks, and 3 weeks all became slower in comparison with post MI cells without GTPs (Figure 2). With the increase of GTPs feeding period the rate of intracellular free radical production was also significantly reduced. In addition, GTPs treatment could help maintain the activity of SOD in cells located at the remote region of the heart in the post MI rats for the time periods from 3 days to 3 weeks, while in post MI group without GTPs treatment the SOD activity was found to be significantly decreased in cardiac tissues of rats suffered from post MI for 3 weeks (Figure 3). However, the measured SOD mRNA level in myocardial tissues was not significantly different in control rats, post MI rats with or without GTPs treatments. For measuring another anti-oxidant enzyme, heme oxygenase-1 (HO-1), the mRNA level was found significantly reduced in cardiac non-infarcted area (remote region) for post MI rats without GTPs treatment, while GTPs treatment prevented from the decrease of mRNA expression in myocardial tissues.

To further examine the events for the oxidative stress in myocardial cells, 4-hydroxynonenal (4HNE) post-translational modification on myocardial proteins were determined in the hearts of control rats, post MI rats with or without GTPs treatments. Results showed that 4-HNE modified proteins were increased in myocardial tissues for the post MI rats without GTPs treatment, but no significant difference with GTPs treatments, as compared to sham controls.

3.4. Ischemic preconditioning cardiac protection signaling pathways

Ischemic preconditioning (IPC) is one of the most effective cardio-protection in which short periods of IR in the heart confer

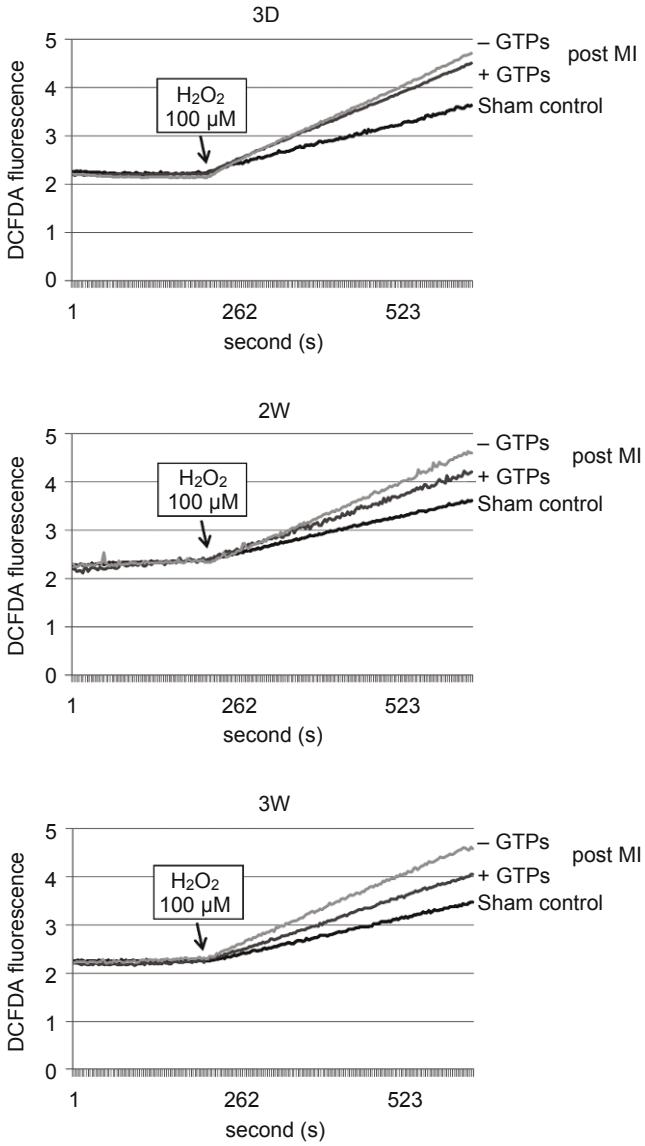


Fig. 2 - The rate of intracellular ROS produced in cardiomyocytes extracted from post MI rats with or without GTPs treatment for 3 days (3D), 2 weeks (2W), and 3 weeks (3W). Intracellular ROS formation was measured by the fluorescence changes of 2',7'-dichlorofluorescin diacetate (DCF-DA) in cardiomyocytes with fluorescence spectrophotometry. The fluorescence excitation maximum for DCF-DA was 495 nm, and the corresponding emission maximum was 527 nm.

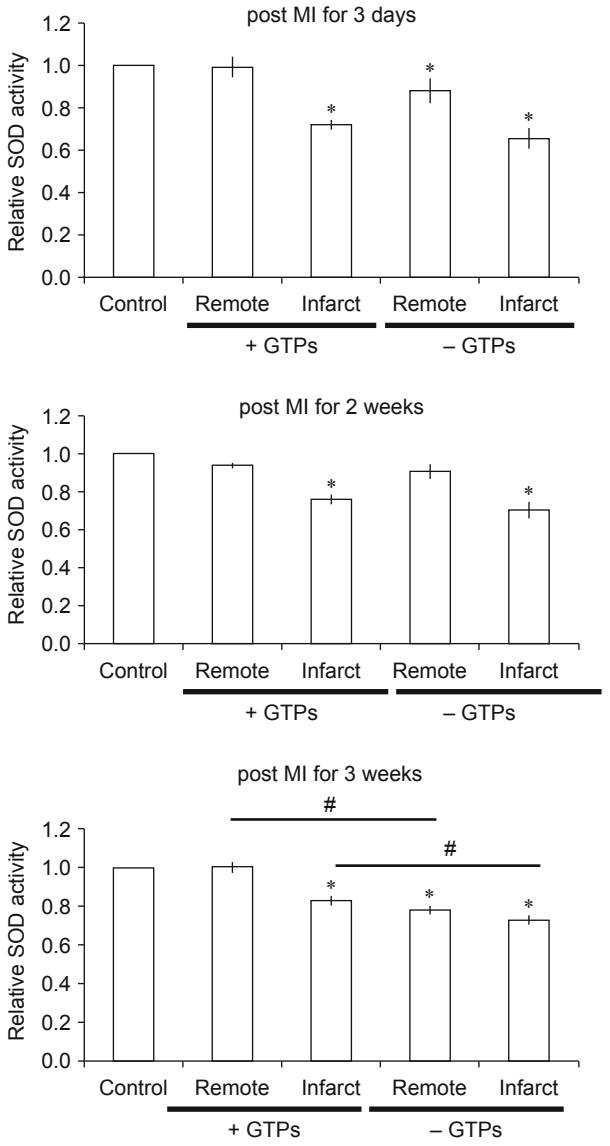


Fig. 3 - The relative activity of SOD in cardiac tissues of sham control, post MI rats with or without GTPs treatment for 3 days, 2 weeks, and 3 weeks.

resistance to a subsequent prolonged ischemic stress [36, 40, 48-51]. Many mediators and effectors have been shown to be essential for IPC and include the ATP-dependent mitochondrial K^+ channels (K_{ATP} channel), PKC, tyrosine protein kinases (TPK), and adenosine, bradykinin, adrenergic and muscarinic receptor, NO donors, and phosphodiesterase inhibitors, and endotoxins, cytokines, and ROS. IPC initiates a number of cardio-protective events depending on the intervening time period between the protective stimulus (IPC) and the index IR injury [36, 40, 50]. Acute IPC (min to hrs) is mediated by the posttranslational modification of proteins, while "second window" IPC (days) induces protection by de novo protein synthesis [51].

Cardiac protection involving a memory (preconditioning) might be attributed to trigger mitochondrial swelling that causes enhanced substrate oxidation and ROS production, leading to redox activation of PKC, which inhibits GSK-3β [52]. Alternatively, TPK or certain G-protein coupled receptor (GPCR)-dependent activation elicits cell protection by inhibiting GSK-3β, *via* Akt and mTOR pathways, PKC pathways, or PKA pathways [44]. The convergence of these pathways *via* inhibition of GSK-3β on the end effector to limit mPTP induction is the general mechanism of cardiomyocyte protection [52]. Recent reports also provided evidence for that the cardio-protection of GTPs against oxidative stress associated with myocardial ischemic injury is caused by reducing cytosolic Ca^{2+} overload and generation of ROS *via* the Akt/GSK-3β/β-catenine and caveolae signaling both *in vivo* myocardial ischemia injury [13, 14] and *in vitro* H_2O_2-induced oxidative stress models [29-30].

3.5. The GPCR-dependent signaling pathways for cardiac protection

The GPCR-dependent mechanism initiates a downstream signaling cascade involving TPK, PI3K/Akt, NOS, activation of K_{ATP} channel, generation of ROS, activation of PKC isoforms, GSK-3β, and MAPK, and inhibition of the opening of the mPTP [49-52]. Although these components are considered to play a role in cardiac protection, it still remains to be resolved as to how signaling networks interact spatially and temporally in producing such protection. In particular, little is known about the regions to which proteins translocate and the molecules with which they interact. In many cases, the signals from GPCRs to target proteins are mediated *via* lipid signals [53].

3.6. Caveolae /lipid rafts involved in cardiac protection

Membrane lipids forms organized and dynamic structures based on interactions between membrane lipids and proteins including caveoli with clear morphology, dynamic rafts of different sizes and specific annular lipid layers surrounding proteins due to mutual affinity of lipids and proteins [54, 55]. It is proposed that these rafts function as platforms for the attachment of proteins when membranes are moved around inside the cell and during signal transduction [54, 55]. It is generally accepted that the structural and functional properties of rafts require an intact microtubule and actin filament; both are the primary interacting partners of caveolae/lipid rafts [56, 57].

Many of the properties of rafts have been inferred from detergent-resistant membranes (DRMs) that occur in nonionic detergent (e.g. Triton X-100) lysates of animal cells [54-55]. Lipid rafts, enriched in cholesterol and sphingolipids, form one such microdomain along with a subset of lipid rafts, caveolae, enriched in the protein caveolin (Cav) [54, 55]. In the membrane raft model, the DRMs represent poorly solubilized rafts [55], and the composition of the DRMs has served as a guide to the structural and functional properties of rafts. These domains selectively and dynamically gather or exclude signaling proteins, and the activity and specificity of membrane proteins is regulated by interaction partners [54, 55]. The "Cav/lipid raft signaling hypothesis" postulates that the regulation of signal transduction events occurs as a result of interaction of signaling proteins with a "Cav scaffolding domain", an interaction that is hypothesized to inhibit such pathways by sequestering components away from signal transduction partners [53-58].

A growing body of data indicates that multiple signal transduction events in the heart occur *via* plasma membrane receptors located in signaling microdomains [59-61]. In the heart, a key Cav is Cav-3, whose scaffolding domain is thought to serve as an anchor for other proteins [62, 63]. Immunoprecipitation with anti-caveolin antibodies indicated that several GPCRs, and their cognate heterotrimeric G proteins and effectors, localize to lipid rafts/caveolae in neonatal cardiac myocytes [64]. Using in vitro and *in vivo* models of IR injury, it has been shown that that the volatile anesthetic, isoflurane, modifies cardiac myocyte sarcolemmal membrane structure and composition and that activation of Src and phosphorylation of Cav-1 contribute to cardiac protection [62, 63]. Thus, multiple signal transduction events in the heart occur *via* plasma membrane receptors located in signaling microdomains [65]. A recent study using *in vitro* oxidative stress model in H9c2 cells has demonstrated that Cav/lipid rafts involve in GTPs-mediated Akt/GSK-3β signaling for cardio-protection during oxidative stress [30]. This study also proposed a hypothetical model with interaction networks based on the identified proteins in EGFP (enhanced green fluorescence protein) expressed cells (Figure 4). It is very likely that GTPs may act to protect cardiac cells from oxidative stress and ischemic injury through lipid rafts.

3.7. Convergence of signaling pathways for cardio-protection on GSK-3β

Numerous cardio-protective drugs are shown to converge on GSK-3β [44, 52, 66-69]. The phosphorylation and inhibition of GSK-3β lead to inhibition or delayed activation of mPTP, a key regulator of apoptosis [52]. A recent study using isolated perfused working rat hearts subjected to global IR demonstrated that addition of a selective inhibitor of GSK-3β prior to ischemia or at the onset of reperfusion improves recovery of left ventricle work by reducing proton production and attenuating the intracellular Ca^{2+} overload [66]. In addition, previous studies have suggested that IPC results in phosphorylation and inhibition of GSK-3β [44], and that drugs that inhibit GSK-3β are cardio-protective [44, 52]. β-catenin is a transcriptional activator that activates target genes in the nucleus [70, 71]. Growth factors promote β-catenin signaling by inhibiting its phosphorylation by GSK-3β, resulting in a reduction of its degradation by the proteasome and its subsequent activation in the nucleus [72, 73]. Cyclin D1 is one of target genes that might be activated by β-catenin for cell proliferation [69]. Consistently, inhibition on the β-catenin signaling pathway would lead to a decrease in cyclin D1 expression in cells that prevent cell cycle progression into S phase [68]. Another route for inhibition of GSK-3β on the β-catenin signaling pathway is to modulate the cell-cell adhesion and communication *via* adherens and gap junction proteins (i.e. Cx43) [13]. Evidence also suggested that GTPs pretreatment acts to protect heart from IR injury through PI3K/Akt survival pathway to limit GSK-3β activity in cardiac cells [13, 29, 30].

4. Molecular targeting for GTPs-mediated cardiac protections

4.1. Cardioproteomics application to discover signaling mechanisms involved in cardiovascular diseases

Proteomics is a new technology that allows the detection and the identification of several proteins at a given time in a sample [74-76]. It combines several techniques, including 2-D gel electrophoresis, image analysis, and mass spectrometry. This technique has been extensively employed to identify proteins involved in cardiac regeneration in the infarcted myocardium [77], to analyze modifications in the plasma protein map during an acute coronary syndrome [78], to analyze the role of complement in myocardial IR and its effect on myocardial protein expression [79]. In an *in vivo* dog model of myocardial IR [80], 2-D gel electrophoresis was used to identify changes in the level of four metabolic enzymes and a contractile protein. In an *in vivo* rabbit model of cardiac IR, Schwertz *et al*. [81] found 10 protein spots that were differentially expressed: two as the protective proteins SOD and αB-crystallin. More recently [82], a proteomic approach was also used to study of the effects of ramipril on post-infarction left ventricular remodeling in the rabbit. In an *in vitro* rat model [83], 8 protein spots with altered expression after cardiac ischemia or IR were found: 5 protein spots as the endoplasmic reticulum enzyme,

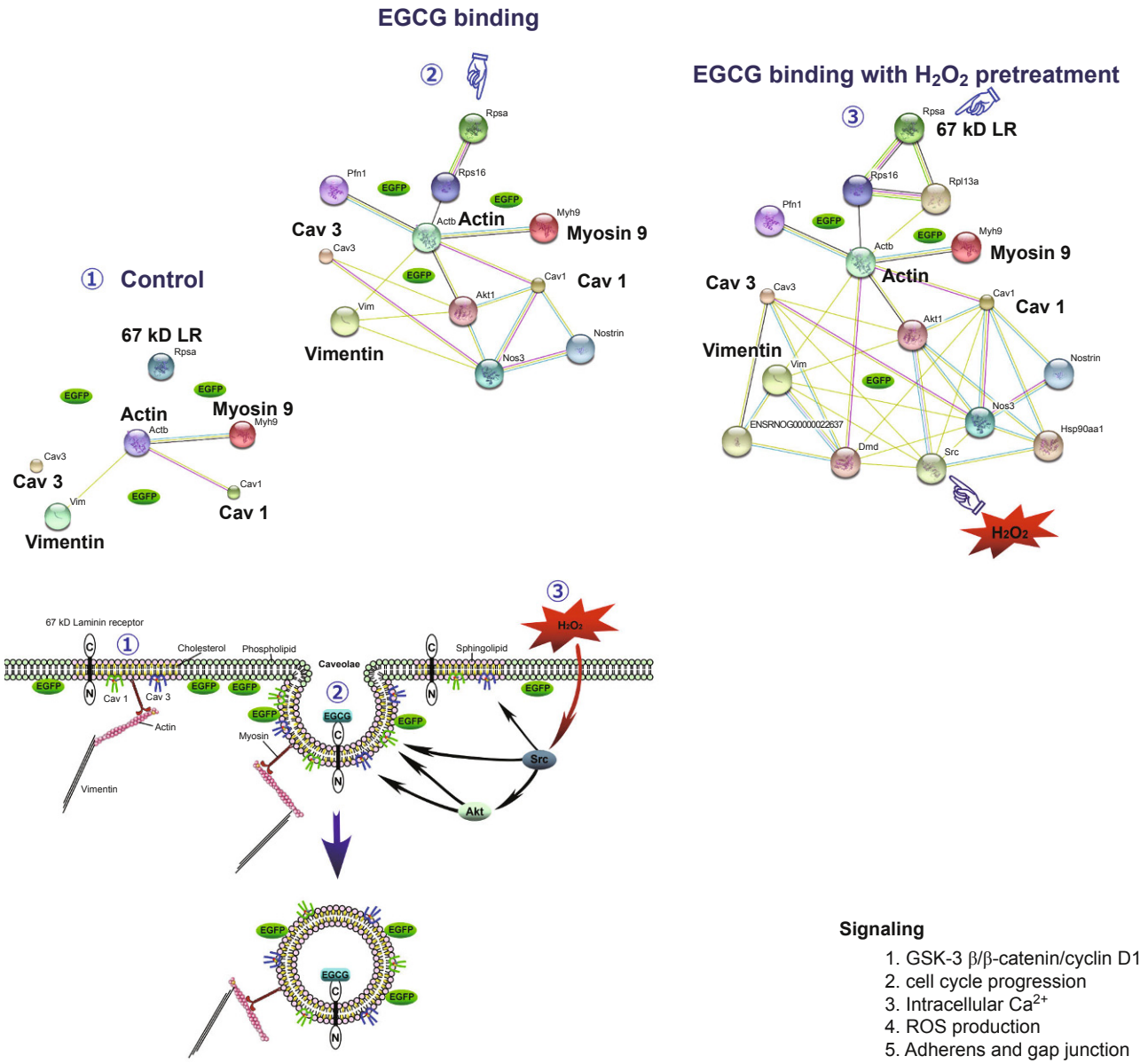


Fig. 4 - A hypothetical model for interaction networks obtained with identified proteins in EGFP-expressing H9c2 cells. The proteins identified were imported into the EMBL Search Tool for the Retrieval of Interacting Proteins (STRING) database, and an interaction map generated was used to construct the hypothetical mechanism for EGCG-induced fluorescence changes in EGFP-expressing H9c2 cells with or without H_2O_2 pretreatment [30].

one as 60 kDa heat shock protein and two as mitochondrial elongation factor Tu. In the mouse, a model of permanent ischemia and a model of IR were used to identify changes in cardiac protein expression after *in vivo* MI by 2-D gel electrophoresis combined with mass spectrometry [84]. Using the H9c2 cell model of H_2O_2-induced oxidative stress for a proteomics study, Chou *et al.* [85] showed that oxidative stress triggers tyrosine phosphorylation on target proteins associated with cell-cell junctions, the actin cytoskeleton, and cell adhesion in cardiac cells.

4.2. Cardioproteomics exploring GTPs-mediated anti-oxidative intervention in H9c2 cardiomyoblasts

To identify the potential proteins for the GTPs-mediated cardioprotection, cardiac proteomics study was performed in an H_2O_2-induced oxidative stress model of myocardial ischemia injury [29]. In this model, 8 proteins associated with metabolism, electron transfer, redox regulation, signal transduction, RNA binding and transcription regulation were identified to take part in EGCG-ameliorating H_2O_2-induced injury to H9c2 cells. H_2O_2 exposure increased oxidative stress evidenced by increases in ROS and cytosolic Ca^{2+} overload, increases in glycolytic protein, α-enolase (Eno 1), decreases in antioxidant protein, peroxiredoxin-4 (Prdx4), as well as decreases in mitochondrial proteins, including aldehyde dehydrogenase-2 (Aldh2), ornithine aminotransferase (Oat), and succinate dehydrogenase ubiquinone flavoprotein subunit (Sdha). All of these effects were reversed by EGCG pre-treatment. In addition, EGCG attenuated the H_2O_2-induced increases of Type II

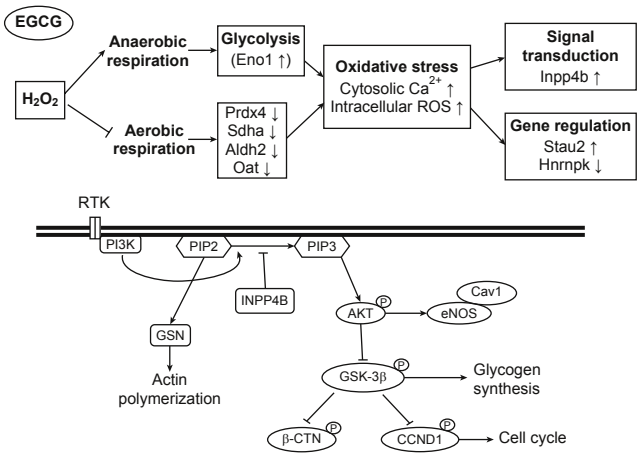


Fig. 5 - The putative mechanism for EGCG-conducted cardio-protection against H_2O_2-induced oxidative stress through the Akt/GSK-3β/caveolae pathways in cultured H9c2 cells. EGCG is hypothesized to protect cardiac cells from oxidative stress by PI3K/Akt survival pathway to attenuate the GSK-3β signaling on cardiac cell death.

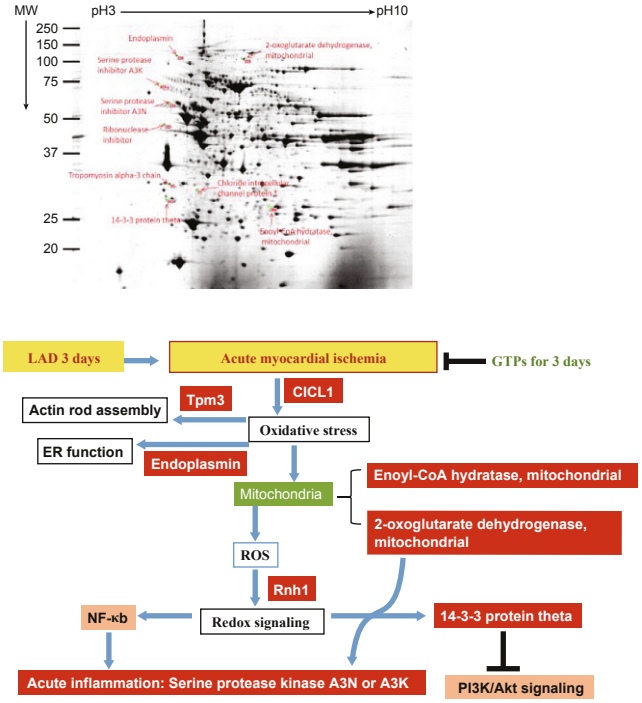


Fig. 6 - Proteomics analysis on molecular identification for targets involved in the GTPs-mediated cardio-protection against AMI in post LAD rats for 3 days.

inositol 3,4-bisphosphate 4-phosphatase (Inpp4b) and relieved its subsequent inhibition of downstream signaling for Akt and GSK-3β/cyclin D1 in H9c2 cells. Pre-treatment with EGCG or GSK-3β inhibitor (SB 216763) significantly improved the H_2O_2-induced suppression on cell viability, phosphorylation of pAkt (S473) and pGSK-3β (S9), and level of cyclin D1 in cells. Finally, EGCG counteracted the H_2O_2-induced decreases in heterogeneous nuclear ribonucleoprotein K (HnrnpK), playing a role in cell cycle progression, but increases in double-stranded RNA-binding protein Staufen homolog 2 (Stau2), involving RNA functions. These findings suggest that GTPs might act to protect cardiac cells from oxidative stress through Akt survival pathway to inhibit the GSK-3β effect on cardiac cell death pathway (Figure 5).

4.3. Cardioproteomics identifying GTPs-mediated cardio-protection against myocardial ischemia stress in post LAD rats

It has been shown that LAD ligation for 3 days caused acute myocardial ischemia (AMI) and impairment of myocardial functions, while myocardial remodeling occurring in the rats after LAD ligation for 2 or 3 weeks [14]. Proteomic analysis has allowed to identify the molecular targets in the myocardium associated with disturbance by ischemia stress but protection by GTPs in post MI rats for 3 days (AMI) or 2-3 weeks (remodeling).

In AMI model associated with post LAD ligation for 3 days, 10 proteins involved in the functions of myocardial ischemia stress (i.e. Chloride intracellular channel protein 1 (CICP1); Endoplasmin), cytoskeletal organization (i.e. Tropomyosin alpha-3 chain, tpm3), mitochondria metabolism (i.e. 2-oxoglutarate dehydrogenase, Enoyl-CoA hydratase), redox signaling (i.e. Ribonuclease inhibitor (Rnh1), 14-3-3 protein θ), and acute inflammation (i.e. Serine protease inhibitors A3N, A3K) were identified for the GTPs-mediated cardio-protection against AMI injury (Figure 6). The data suggested that the activation of NF-κb transcription factor and inhibition on PI3K/Akt signaling might account for the AMI-induced stress, and such redox signaling events could be prevented by GTPs (Figure 6).

In remodeling model with post LAD ligation for 2 weeks, 14 proteins associated with chaperone proteins (i.e. Heat shock protein 75 kDa, mitochondrial; 60 kDa heat shock protein, mitochondrial), muscle proteins (i.e. cardiac troponin T, desmin), lipid metabolism (i.e. Carnitine O-acetyltransferase), mitochondria functions (i.e. ES1 protein homolog, Electron transfer flavoprotein subunit β, Fumarate hydratase, ATP synthase subunit α, ATP synthase subunit β, inner membrane protein fragment), developmental protein (i.e. dihydropyrimidinase-related protein 2), and stress related adaptor protein (i.e. 14-3-3 protein ε) were identified for the GTPs-mediated cardio-protection against the myocardial remodeling after ischemia stress (Figure 7). These data suggest that during myocardial ischemia remodeling cardiac cells disturbed by mitochondria dysfunction associated with alterations of lipid metabolism trigger chaperone/stress response *via* the adaptor protein (14-3-3 protein ε) resulting in cytoskeleton reorganization and contractile apparatus disruption. Such stress-induced redox signaling for myocardial ischemia remodeling could be improved by GTPs. Consistently, myocardial remodeling with post LAD for 3 weeks also identified 10 proteins associated with cytoskeletal function, energy metabolism (i.e. electron transport chain, citric acid cycle, and fatty acid oxidation), and redox regulation (Figure 8).

5. Perspectives

Green tea, being rich in polyphenols (GTPs), is a natural choice for its myocardial protection against ischemia or oxidative stress. Cardiac proteomics have allowed to reveal the underlying mechanisms for the actions of GTPs exerting their favorable cardio-protective effects. Currently, the important findings illustrating

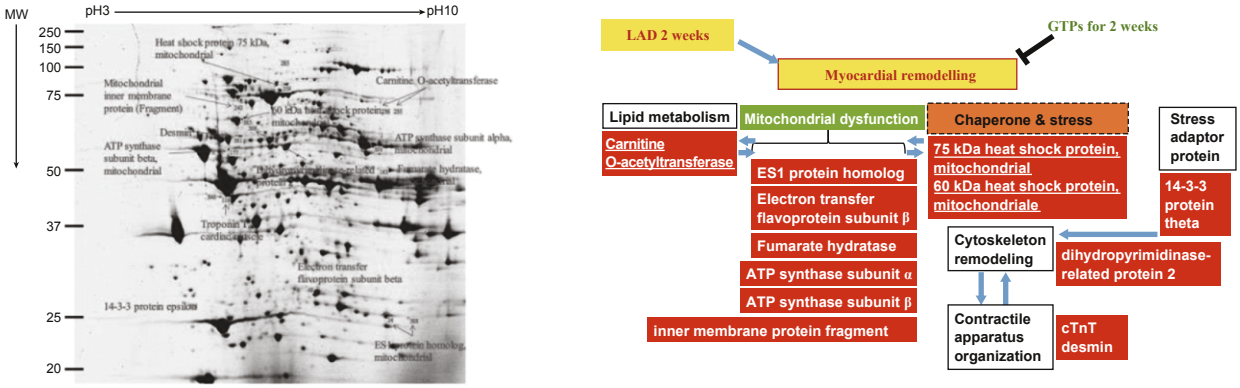


Fig. 7 - Proteomics analysis identifying molecular targets involved in the GTPs-mediated cardio-protection against myocardial remodeling in post LAD rats for 2 weeks.

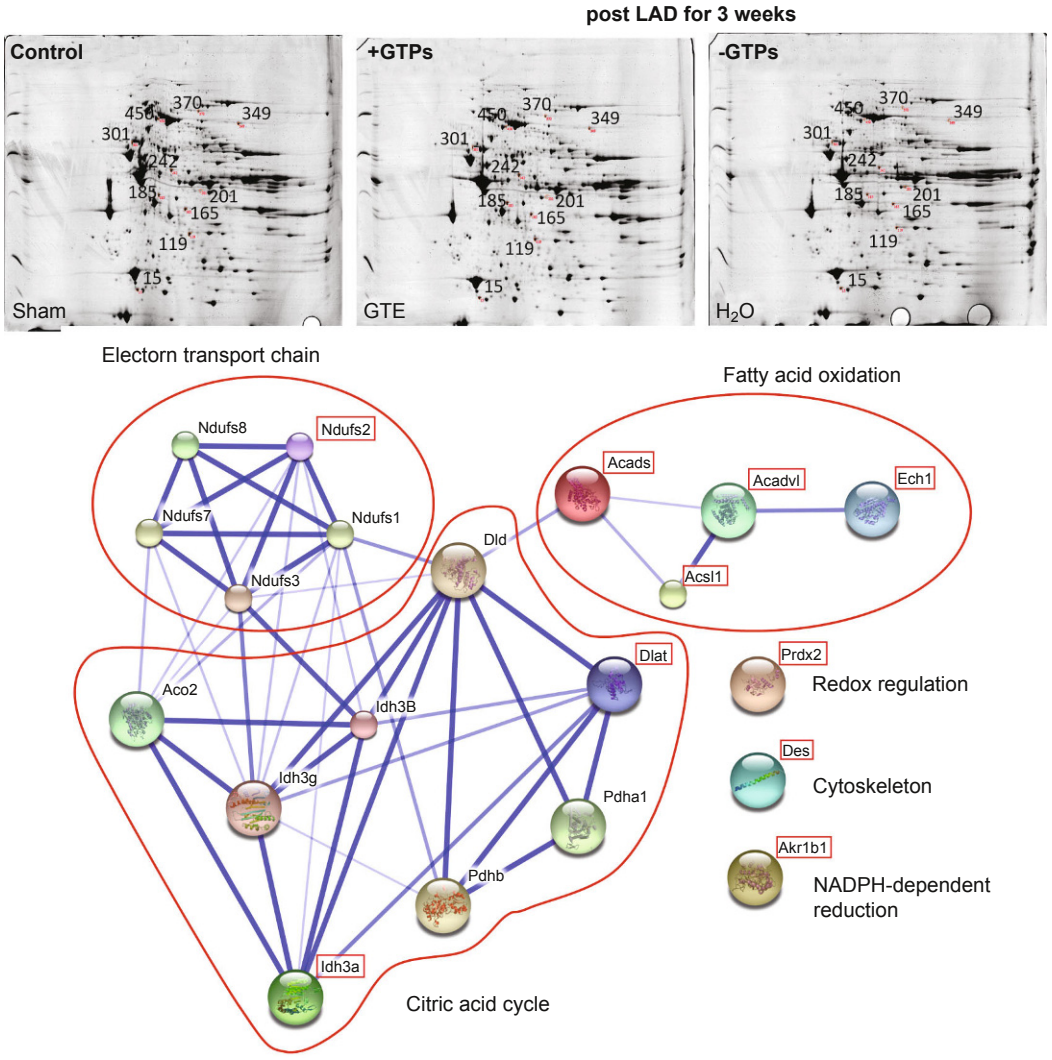


Fig. 8 - Molecular identification and hypothetical protein-protein interactions for proteins involved in myocardial remodeling of post MI for 3 weeks with or without GTPs in rats. Identified proteins are *Peroxiredoxin-2 (Prdx2) (Spot 15), Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial, (Ech1) (Spot 119), Aldose reductase (Akr1b1) (Spot 165), Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial (Idh3a) (Spot 185), Short-chain specific acyl-CoA dehydrogenase, mitochondrial (Acads) (Spot 201), NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial (Ndufs2) (Spot 242), Desmin (Des)(Spot 301), Very long-chain specific acyl-CoA dehydrogenase, mitochondrial (Acadvl) (Spot 349), Long-chain-fatty-acid--CoA ligase 1 (Acsl1) (Spot 370), Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial (Dlat0 (Spot 450).*

the potential end effectors involved in cardiac protection by GTPs include: (1) EGCG exerting cardio-protection against H_2O_2-induced oxidative stress through the Akt/GSK-3β/caveolae pathways in cardiac cells, (2) GTPs preventing their activation of NF-κb and their inhibition on PI3K/Akt signaling for the AMI stress, (3) GTPs ameliorating mitochondria dysfunction associated with alterations of lipid metabolism, chaperone-induced stress response, and the adaptor 14-3-3 ε protein signaling for cytoskeleton remodeling /contractile apparatus disruption during post MI remodeling. It appears promising to apply this natural product as a therapeutic approach to treat ischemic heart diseases in the near future.

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7. Conflicts of interest statement

The authors declare that they have no conflicting interests.

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Review article

New mechanisms of antiplatelet activity of nifedipine, an L-type calcium channel blocker

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ABSTRACT

Platelet hyperactivity often occursd in hypertensive patients and is a key factor in the development of cardiovascular diseases including thrombosis and atherosclerosis. Nifedipine, an L-type calcium channel blocker, is widely used for hypertension and coronary heart disease therapy. In addition, nifedipine is known to exhibit an antiplatelet activity, but the underlying mechanisms involved remain unclear. Several transcription factors such as peroxisome proliferator-activated receptors (PPARs) and nuclear factor kappa B (NF-κB) exist in platelets and have an ability to regulate platelet aggregation through a non-genomic mechanism. The present article focuses on describing the mechanisms of the antiplatelet activity of nifedipine *via* PPAR activation. It has been demonstrated that nifedipine treatment increases the activity and intracellular amount of PPAR-β/-γ in activated platelets. Moreover, the antiplatelet activity of nifedipine is mediated by PPAR-β/-γ-dependent upon the up-regulation of the PI_3K/AKT/NO/cyclic GMP/PKG pathway, and inhibition of protein kinase Cα (PKCα) activity *via* an interaction between PPAR-β/-γ and PKCα. Furthermore, suppressing NF-κB activation by nifedipine through enhanced association of PPAR-β/-γ with NF-κB has also been observed in collagen-stimulated platelets. Blocking PPAR-β/-γ activity or increasing NF-κB activation greatly reverses the antiplatelet activity and inhibition of intracellular Ca^{2+} mobilization, PKCα activity, and surface glycoprotein IIb/IIIa expression caused by nifedipine. Thus, PPAR-β/-γ-dependent suppression of NF-κB activation also contributes to the antiplatelet activity of nifedipine. Consistently, administration of nifedipine markedly reduces fluorescein sodium-induced vessel thrombus formation in mice, which is considerably inhibited when the PPAR-β/-γ antagonists are administrated simultaneously. Collectively, these results provide important information regarding the mechanism by which nifedipine inhibits platelet aggregation and thrombus formation through activation of PPAR-β/-γ-mediated signaling pathways. These findings highlight that PPARs are novel therapeutic targets for preventing and treating platelet-hyperactivity-related vascular diseases.

1. Introduction

Platelets are unnucleated fragments derived from bone marrow megakaryocytes. Traditionally, the most well-known function of platelets is that they are responsible for hemostasis in response to vascular injury and endothelial disruption. Recent studies have indicated that platelets also have an immunomodulatory activity through production of several pro-inflammatory mediators promoting pathogenic thrombi formation and inflammatory responses [1, 2]. Platelets perform their functions mainly through secretion of several proteins stored in various cytoplasmic granules. There are at least three different types of granules (α-granules, dense core granules, lysosomes), and a complex membranous system in platelets. The α-granules contain hemostatic factors (factor V, von Willebrand factor (vWF) and fibrinogen) and other cytokines, mitogenic factors (PDGF and bFGF) and proteases (MMP2, MMP9) [3]. The mediators stored in α-granules can be selectively released in response to the activation of different receptors. Dense granules store small non-protein molecules such as ADP, ATP, serotonin, calcium and pyrophosphate, which all play a central role in the amplification of platelet aggregation. Lysosomes contain glycosidases, proteases, and cationic proteins with bactericidal activity.

Excessive platelet activation has been regarded as a key pathological factor in the development of many vascular diseases such as acute coronary syndromes, myocardial infarction and atherothrombosis [4, 5]. Endothelial dysfunction/injury initially induces platelet activation, and promoting their interaction with neutrophils and monocytes leads to the pathogenesis of atherosclerosis. Therefore, platelets are an important link between tissue damage and hemostatic and inflammatory responses. In supporting this

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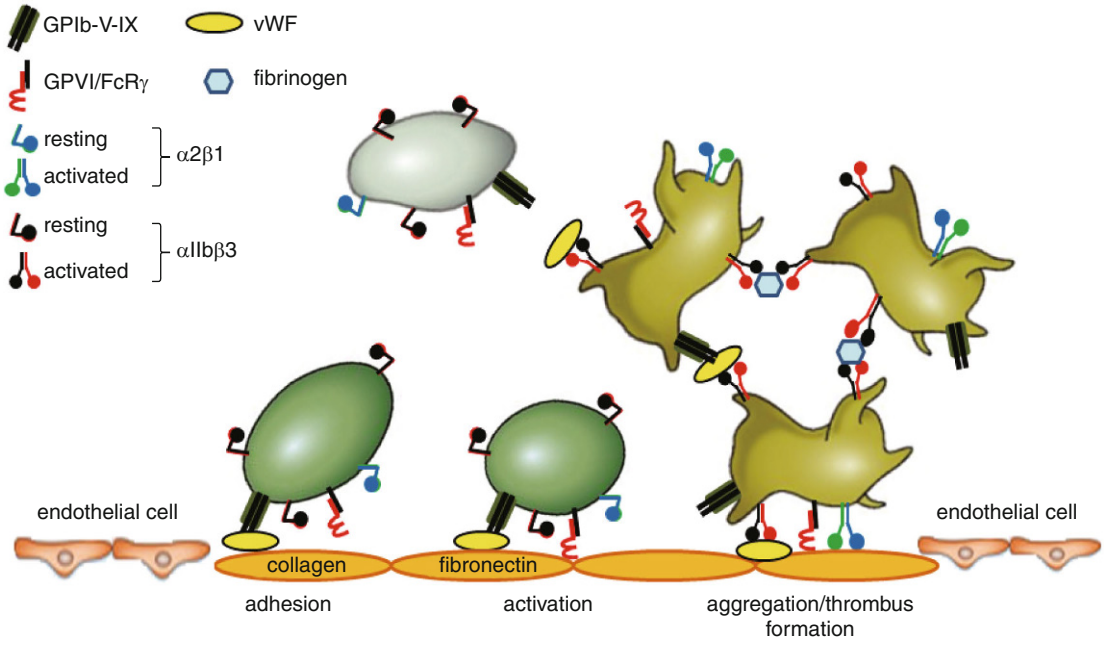

Fig. 1 - Platelet adhesion and aggregation at the sites of vascular injury. The interaction of GPIb-V-IX and vWF enables GPVI binding to collagen in injured vessels. This triggers platelet aggregation by crosslinking adjacent platelets through binding to fibrinogen and vWF *via* the activated GPIIb–IIIa receptors.

concept, several lines of evidence have demonstrated that platelet hyperactivity often occurs in hypertensive or cardiovascular patients [6, 7]. Thus, agents with inhibiting platelet hyperactivity may be potential therapeutic drugs for platelet-related vascular diseases.

2. Platelet activation

Platelet adhesion to the extracellular matrix is the initial step in haemostasis [8]. When vascularity is damaged, the immobilized vWF on exposed collagen becomes a strong adhesive substrate. The vWF, a multimeric adhesive glycoprotein, contains binding sites for collagen glycoprotein (GP)Ib and integrin GPIIb/IIIa (αIIbβ3) [9]. The adhesion is mediated by the interaction between the GPIb-IX-V receptor complex on the platelet surface to vWF, and GPVI and GPIα to collagen at sites of vascular injury. The interaction of vWF and GPIb-IX-V complex is required for the adhesion of platelets to the subendothelium, which enables GPVI binding to collagen [10]. In addition, collagen serves as a binding site for vWF in the subendothelial matrix, and therefore contributes to the adhesion of unactivated platelets via GPIb-IX-V (Figure 1) [11]. The adhesion is followed by platelet aggregation by binding to soluble fibrinogen and vWF *via* the activated integrin GPIIb/IIIa. Collectively, upon activation of the glycoprotein receptors, it promotes platelet adhesion, aggregation, and spreading on the exposed extracellular matrix of the injured vessel wall, as well as thrombus formation and stability [12].

There are multiple pathways regulating platelet activation. The platelet agonists including ADP, thrombin, collagen and serotonin perform their functions through their specific receptors of platelets. ADP stored in dense granules is released during platelet activation. ADP promotes platelet activation through its receptors ($P2Y_1$ and $P2Y_{12}$). $P2Y_1$ is a G-protein-coupled seven-transmembrane domain receptor that stimulates platelet shape change and mobilizes calcium from intracellular stores by activating phospholipase C (PLC) [13]. Activation of the $P2Y_{12}$ receptor inhibits the adenylate cyclase activity of platelets and seems to be responsible for a positive feedback mechanism for platelet stimulation especially by weak agonists [14]. There are two receptors (protease activated receptors 1 (PAR1) and 4 (PAR4)) on platelet surfaces for thrombin, the most potent physiological platelet activator [15]. PAR1 mediates platelet activation at low concentrations of thrombin, while PAR4 is activated at higher thrombin concentration. PARs are also expressed in other cells in the vasculature, cells such as leukocytes, endothelial cells and smooth muscle cells. It has been reported that PAR-1 and PAR-2 expressed in the vessel wall are involved in contractility, inflammation, proliferation, and repair [16]. Collagen is also a strong platelet activating agent because it stimulates platelet adhesion and is mediated by the binding of vWF to it at the sites of vascular injuries. Moreover, other glycoprotein receptors such as GPIb, GPIIb/IIIa, GPIa/IIa, and GPVI all contribute to collagen-mediated platelet activation [1]. Serotonin expresses its actions through the serotonin receptor 5-HT2A to enhance procoagulant activity *via* retention of fibrinogen and thrombospondin on platelet surfaces. Furthermore, epinephrin and other catecholamines stimulate the platelet α2A-adrenergic receptor, which coupled with a G-protein leads to the inhibition of adenylate cyclase activity, which in turn induces platelet aggregation [17].

When platelets are activated by collagen or thrombin, arachidonic acid (AA) is liberated from membrane phospholipids. This liberation is caused by Ca^{2+}-dependent phospholipase A_2 (PLA_2), diglyceride lipase and/or phosphatidic acid specific phospholipase A_2 [18]. Then, AA is converted to thromboxane A_2 (TXA_2), a potent activator for the release reaction and aggregation of platelets by cyclooxygenase (COX) and thromboxane synthase. TXA_2, when released from the platelets, binds to the G-protein coupled thromboxane receptor and functions as an agonist for platelet activation

[19]. In addition, TXA_2 also participates in a positive feedback loop further increasing $[Ca^{2+}]_i$ and AA-induced ATP release and platelet aggregation [20]. Phosphoinositides breakdown is another critical mechanism accounting for agonist-induced platelet activation. Simulation by agonists such as thrombin or collagen results in PLC-catalyzed hydrolysis of plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate, with concomitant formation of inositol 1,4,5,-trisphosphate (IP_3) and diacylglycerol [21]. The IP_3 binds to the IP_3 receptor on sarcoplasmatic reticulum (SR) triggering a significant release of Ca^{2+} from SR, leading to an increase of intracellular Ca^{2+} concentration [22]. It has been demonstrated that an increase of intracellular Ca^{2+} concentration, as a result of either calcium influx and/or calcium release from intracellular stores, is fundamental to platelet activation [23]. Importantly, diacylglycerol acts as an activator for protein kinase C (PKC) that is essential for agonist-induced platelet aggregation and granule secretion [24]. At least seven PKC isoforms (α, β, δ, θ, ε, η, and ζ) are found in platelets. Among these PKC isoforms, activation of PKCα through Syk-dependent phosphorylation is crucial for platelet activation [25]. Moreover, TXA_2 has an ability to activate PKC by activating PLC-dependent pathways [26], suggesting that blocking TXA_2 formation may inhibit PKCα-related signaling pathways. Mitogen-activated protein kinases (MAPKs) containing extracellular signal-regulated kinase (ERKs), the c-Jun N-terminal kinase (JNK) and p38 MAPK have been identified in platelets [27]. The roles of JNKs and ERKs in platelet mechanisms are still unclear. On the other hand, activation of p38 MAPK by PKC can phosphorylate $cPLA_2$ on Ser505, leading to the production of AA and TXA_2 synthesis [28]. Thus, $cPLA_2$ activation is regulated, at least in part, by PKC through p38 MAPK. These platelet-activating mechanisms ultimately up-regulate surface GPIIb/IIIa expression, thereby promoting the binding of fibrinogen and platelet aggregation.

3. Antiplatelet mechanisms

3.1. NO/cyclic GMP

Nitric oxide (NO), synthesized from L-arginine by nitric oxide synthase (NOS), activates intracellular soluble guanylyl cyclase (sGC) and guanosine 3',5'-cyclic monophosphate (cGMP) formation, which subsequently activates cGMP-dependent protein kinase (PKG). The NO-dependent signaling pathway is known to play an important modulatory role both in physiological and pathological conditions [29]. Up-regulation of the NO/cGMP/PKG1 cascade reportedly inhibits platelet activation by regulating actin filament dynamics, integrin activation, and intracellular Ca^{2+} mobilization, which in turn suppresses PLC and PKC activity [30, 31]. Moreover, PKG promotes sarcoplasmic reticulum ATPase (SERCA)-dependent refilling of intraplatelet Ca^{2+} stores and inhibits inositol-1,4,5-trisphosphate-stimulated Ca^{2+} release from the sarcoplasmic reticulum, which in turn decreases intracellular Ca^{2+} level and platelet activation [30]. PKG is capable of phosphorylating the TxA_2 receptor, thereby inhibiting its function. cGMP also indirectly increases intracellular cAMP through inhibition of phosphodiesterase type 3 to synergistically inhibit platelet aggregation [33]. Interestingly, previous studies have indicated that the actions of NO on platelet function are also mediated by a cGMP-independent mechanism that inhibits exocytosis of platelet granules (dense, lysosomal, and α-granules) by S-nitrosylation of N-ethylmaleimidesensitive factor (NSF) [34].

3.2. Cyclic AMP

It has been demonstrated that the elevation of cyclic AMP formation reduces platelet functions including adhesion, aggregation, the release of granule contents as well as the rise of intracellular Ca^{2+} mobilization [33, 35]. The steady-state level of cyclic AMP is maintained by a balance between the rate of synthesis by adenylate cyclase and the rate of degradation by cyclic AMP phosphodiesterase. Research has shown that several cyclic AMP-elevating agents exert antiplatelet activity through a cyclic AMP-dependent protein kinase (PKA)-dependent signal pathway [36, 37].

3.3. GP IIb/IIIa Inhibitors

When platelets are activated by physiological agonists such as thrombin, ADP, or collagen, the intracellular signal pathways for platelet activation are stimulated and thereby induce conformational changes of αIIbβ3 leading to the formation of an activated state with high affinity for fibrinogen and numerous other ligands [1, 38]. As a result of the enhancement of αIIbβ3-mediated binding to the bivalent molecule, fibrinogen may cause platelets aggregation. To date, two binding sites have been well characterized in αIIbβ3: an Arg-Gly-Asp (RGD)-binding site and a Lys-Glu-Ala-Gly-Asp-Val (KQAGDV)-binding site. Fibrinogen binds *via* the KQAGDV-binding site. Agents that bind within the ligand-binding region of αIIbβ3 and block the binding of its natural ligands have been developed and termed GPIIb/IIIa inhibitors. There are three FDA-approved integrin αIIbβ3 inhibitors, and they include abciximab (ReoPro; Lilly), eptifibatide (Integrilin; millennium Pharmaceuticals/Schering–Plough), and tirofiban (Aggrastat; merck). Abciximab is a murine human chimeric fab fragment that was derived from the murine monoclonal antibody 7E3. Eptifibatide is a KGD-containing cyclic heptapeptide. And tirofiban is a non-peptide derivative based on the RGD sequence [39]. Clinical studies have indicated that a blockade of the glycoprotein IIb/IIIa receptors limits the inflammatory responses secondary to coronary intervention, suggesting that inhibition of inflammatory marker expression by GPIIb/IIIa inhibitors may contribute to its clinical benefit [40]. Our previous study tested the effect of the synthesized RGRHGD with the highest local hydrophilicity region of B chain of β-bungarotoxin on platelet aggregation. The RGRHGD holds parts of both RGD and KGD peptides that have been reported to exhibit a high binding affinity to GPIIb/IIIa. Moreover, the inhibitory effect of RGRHGD on platelet aggregation is associated with attenuation of TXA_2 formation and intracellular calcium mobilization. These findings may, at a later date, provide a useful method for finding potential therapeutic agents through molecular modeling analysis [41].

4. Peroxisome proliferator-activated receptors (PPARs) and platelet activation

PPARs belonging to ligand-activated transcription factors modulate several important biological effects, including lipid, glucose homeostasis, energy metabolism, and inflammation [42, 43]. A variety of compounds can serve as PPAR ligands and activate the receptor. PPARα ligands are fatty acids, and their derivatives as well as eicosanoids include 8-S-hydroxyeicosatetraenoic acid (8SHETE) and leukotriene B4 (LTB4) [44]. Moreover, fibrates, the synthetic ligands for PPARα, are widely used in the treatment of hypertriglyceridemia and hyperlipidemia [44]. Other phar-

macological compounds such as nonsteroidal anti-inflammatory drugs (NSAIDs) are confirmed as PPARα ligands [45]. PPARγ is activated by 15-deoxy-12, 14-prostaglandin J2 (PG-J2) and 15-hydroxyeicosatetraenoic acid (15-HETE) [46, 47] that are AA metabolites derived from COX and lipoxygenase pathways. In addition, fatty acid-derived compounds of oxidised LDL, including 9- and 13-hydroxyoctadecadienoic acid (9- and 13-HODE), and glitazones [48] (an antidiabetic drug), indomethacin, and ibuprofen all function as ligands for PPARγ [45]. In response to their ligands, PPARs undergo a conformational change leading to the recruitment of distinct coactivators and corepressors. Subsequently, these changes result in PPAR heterodimerization with cis-retinoid X receptor (RXR) and in turn regulate downstream gene expression by binding to the peroxisome proliferator response element (PPRE) that exist in the promoter of target genes of nucleated cells. Although platelets are anuclear cells, they also contain transcription factors such as PPARs. The existence of three PPAR isoforms (α, β/δ, and γ) in human platelets has been demonstrated, and activation of PPARs inhibits platelet aggregation through a nongenomic mechanism [49, 50]. It has been reported that the inhibitory effect of PPAR agonists on platelet aggregation is associated with the modulation of GPVI, PKCα and calcium mobilization signals [51]. Our recent study indicated that the antiplatelet activity of alpha-lipoic acid is mediated by PPAR-α/-γ-dependent processes [50]. Therefore, reagents exerting PPAR-activating activity have been regarded as a new class of antiplatelet drugs.

5. PPARs and atherosclerosis

Atherosclerosis is a complex process characterized by lipid accumulation in the arterial wall resulting in heart and brain infarction. The atherogenesis is initiated *via* the attraction of various cells such as monocyte/macrophages, T lymphocytes, endothelial cells, and smooth muscle cells (SMCs). This cellular activation promotes local inflammatory responses and migration and proliferation of SMCs, which in turn leads to the formation of foam cells. PPARs are expressed in the vascular wall and atherosclerotic lesions, suggesting that they may modulate the atherogenic processes. Clinical studies have indicated that PPARγ ligand troglitazone inhibits SMC proliferation and decreases the intima and media thickness of carotid arteries [52]. The inhibitory effects of PPARs (α and γ) on the expression of inflammatory genes, such as interleukin-6, cyclooxygenase-2, inducible nitric oxide synthase (iNOS), matrix metalloproteinase-9, endothelin-1, lipid accumulation within the plaque, and thrombogenesis [53] have been proposed to be the underlying mechanisms for their anti-atherosclerotic effects. Furthermore, PPARα activators also can induce apoptosis of activated macrophages by inhibiting the antiapoptotic NF-κB pathway [54] and reducing monocytic recruitment to early atherosclerotic lesions by inhibition of monocyte-recruiting proteins such as vascular cell adhesion molecules (VCAM)-1 expression in endothelial cells [55]. These findings suggest that up-regulation of PPAR expression/activation may prevent the progress of atherosclerotic disease.

6. Nifedipine, a dihydropyridine calcium channel blocker (CCB)

The calcium channel blockers (CCB) are a group of drugs used to treat cardiovascular diseases including hypertension, angina, and peripheral vascular disorders. CCBs were approved for the treatment of hypertension in the 1980s. Since then, CCBs have increased markedly because of their effective lowering of blood pressure with few side effects. In addition to the cardiovascular effects of CCBS, other beneficial functions of CCBs, such as antioxidative, anti-inflammatory, anti-atherosclerotic, bone-remodeling, and immunomodulating properties have been reported [56]. It is well known that inflammation is a fundamental basis of atherosclerosis. Several reports have revealed that dihydropyridine CCBs exert anti-inflammatory effect by suppressing the tumor necrosis factor (TNF), monocyte chemoatractant protein-1 (MCP-1), and pro-inflammatory cytokine expression accompanied by the reduction of NF-κB activation in various vascular cells including endothelial cells, macrophages, and smooth muscle cells [56]. These actions of CCBs may contribute to the anti-atherosclerotic effect in vascular cells.

CCBs can be classified into 3 main classes according to their different structure. The three classes are the phenylalkylamines (e.g., verapamil), the benzothiazepines (e.g., diltiazem), and the dihydropyridines (e.g., nifedipine, amlodipine, isradipine). It is known that different classes of CCBs have differing pharmacologic actions. With their relative potency of lowering blood pressure, the dihydropyridine-type compounds such as nifedipine are the most potent subclass. Nifedipine, a dihydropyridine-based L-type CCB, is widely used in the treatment of hypertension and coronary heart diseases. Clinical studies have shown that a significant reduction of new coronary lesions and the intima-media thickness in the carotid artery were observed in patients treated with nifedipine [57]. These findings confirmed that nifedipine has an anti-atherosclerotic effect beyond its blood pressure-lowering effect. The protection may be associated with suppressing reactive oxygen species (ROS) formation and subsequent inflammatory responses, as well as smooth muscle cell proliferation, migration and differentiation [56]. Importantly, nifedipine can activate PPAR-γ by inhibiting ERK1/2 activity in macrophages [58], suggesting that PPARs may involve the pharmacological effects of nifedipine. As increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is essential for platelet activation, therefore, reagents that attenuate platelet $[Ca^{2+}]_i$ may have an antiplatelet activity. As expected, nifedipine is capable of inhibiting platelet aggregation, though platelets lack L-type calcium channels. To date, the underlying molecular mechanisms remain unclear; as although the activation of NO/cGMP-dependent signaling pathway [59, 60] has been proposed as a possible mechanism that contributes to its antiplatelet activity of CCBs.

7. Nifedipine-mediated anti-aggregatory effect *via* activation of PPAR-β/-γ

PPARs play an important role in the modulation of metabolism and inflammatory processes, and exhibit a protective effect against the development of atherosclerosis and cardiovascular diseases [61, 62]. Traditionally, the actions of transcription factors are thought to be mainly attributed to their regulation in gene expression. Recently, accumulating evidence supports that there are nongenomic actions of these receptors [63]. Because a number of transcription factors including PPARs, estrogen receptors (ER), and nuclear factor kappa B (NF-κB) have been found in platelets, many studies have focused on the role of cytoplasmic PPARs in platelet function. It has been reported that the activation of

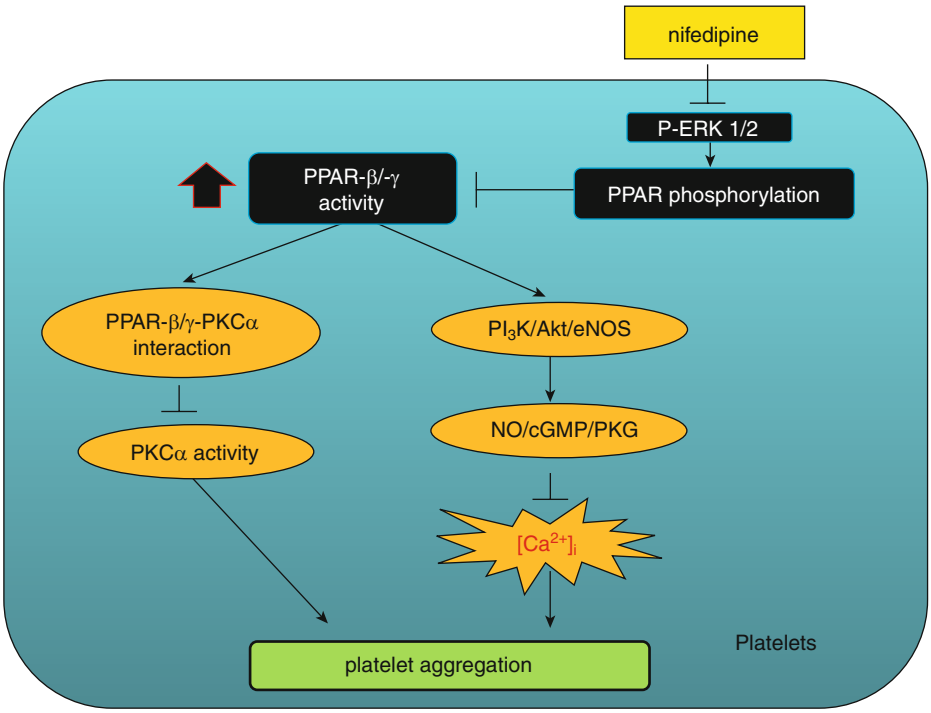

Fig. 2 - The antiplatelet activity of nifedipine is mediated by PPAR-β/γ. Nifedipine increases the activity and intracellular levels of PPAR-β/γ in activated platelets. Subsequently, PPAR-β/-γ-dependent up-regulation of PI_3K/Akt/eNOS/NO/cyclic GMP/PKG cascade, inhibition of PKCα activity *via* association of PPAR-β/γ with PKCα, and intracellular Ca^{2+} mobilization ultimately inhibits platelet aggregation.

PPARs (α, β/δ, and γ) by respective agonists inhibits platelet aggregation [49, 50] and slows intraarterial thrombus formation due to increased NOS expression [64]. Thus, selective ligands for PPARs may negatively regulate platelet activation. Based on the finding that nifedipine induces PPAR-γ activation in macrophages and smooth muscle cells [58], it is possible that PPARs may involve nifedipine-mediated regulation of platelet and vascular functions.

Our recent study has confirmed that treatment with nifedipine significantly increases PPAR-β and PPAR-γ activity due to the inhibition of phosphorylation of ERK1/2 and PPAR-γ without affecting PPAR-α activity in collagen-stimulated platelets [65]. These results indicat that nifedipine is a dual PPAR-β/-γ activator in platelets. However, other CCBs like amlodipine are PPAR-β activators, and lacidipine has no significant effect on PPARs activity in human platelets, suggesting that the activation of PPARs is not a common effect of all CCBs. Accordingly, the effects of different CCBs on platelet PPARs activity and the role of PPARs on CCBs-mediated antiplatelet activity are diverse and chemical structure specific. Upon activation by inducers such as collagen, a rapid release of PPAR-β/-γ from the α-granules of platelets into extracellular regions results in a marked reduction of the intracellular amount of PPAR-β/-γ, which may have a systemic effect. A novel finding is that nifedipine greatly inhibits the release of PPAR-β/-γ from activated platelets, thereby increasing the intracellular availability of PPAR-β/-γ which may enhance its cellular functions like the regulation of platelet activation. However, the underlying mechanisms accounting for the phenomenon require further investigation. Blocking PPAR-β/-γ activity with their specific antagonists significantly reverss the inhibitory effect of nifedipine on platelet aggregation, supporting that PPAR-β/-γ is involved in the antiplatelet activity of nifedipine. In addition, nifedipine-mediated up-regulation of the PI_3K/Akt NO/cGMP/PKG cascade that results in a reduction of Ca^{2+} mobilization is regulated by a PPAR-β/-γ-dependent signaling pathway (Figure 1). The activation of PKCα is crucial for platelet secretion and aggregation [24]. Consistent with our previous findings is the discovery that the inhibitory effect of PPAR-α/-γ on platelet PKCα activity is associated with its association with PKCα [50]. In collagen-stimulated platelets, nifedipine also induces an interaction between PPAR-β/-γ and PKCα accompanied by decreased PKCα activity evidenced by reduced PKCα phosphorylation in the complex. Similarly, an addition of PPAR-β/-γ antagonists abrogated the attenuation of PKCα activity by nifedipine, suggesting that direct interaction between PPAR-β/-γ and PKCα is a possible way to suppress PKCα activity.

Furthermore, administration of nifedipine markedly inhibited fluorescein sodium and irradiation-induced vessel thrombus formation *in vivo*. However, the antithrombotic effect of nifedipine was considerably reduced when PPAR-β/-γ antagonists were administrated simultaneously. Taken together, the antiplatelet and antithrombotic effects of nifedipine are mediated by activation of PPAR-β/-γ leading to up-regulation of the NO/cGMP/PKG cascade, as well as inhibition of PKCα activity and intracellular Ca^{2+} mobilization.

8. NF-κB and platelet activation

NF-κB, a transcription factor, normally exists as an inactive cytoplasmic complex heterodimer complex composed of p50 and p65 subunits bound to the inhibitory protein, IκB-α. Upon stimula-

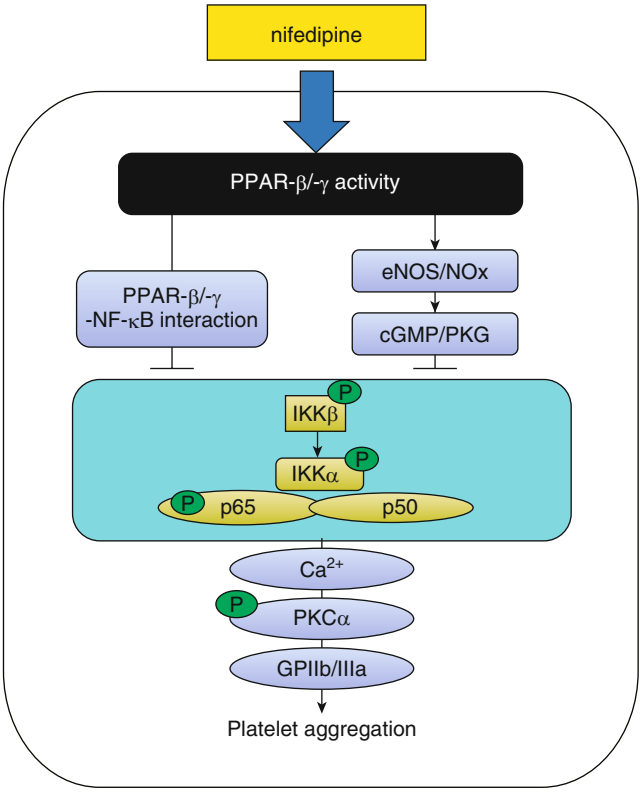

Fig. 3 - PPAR-β/-γ-dependent inhibition of NF-κB activation involves the antiplatelet activity of nifedipine. The decreased NF-κB activation accompanied by reduction of phosphorylation of IKK, IκBα, and p65NF-κB by nifedipine is mediated by a direct association of PPAR-β/-γ with NF-κB and PPAR-β/γ-dependent up-regulation of the NO/cyclic GMP/PKG1 pathway. This attenuates subsequent intracellular Ca^{2+} mobilization, PKCα activation, and surface GPIIb-IIIa expression, which in turn inhibits platelet activation.

tion, IκB-α is phosphorylated by IκB kinases (IKKs) leading to rapid degradation by proteasome and the subsequent release of NF-κB from its inhibitors. Then, the free NF-κB translocates to the nucleus, where it activates the transcription of inflammation-related target genes [66]. The activation of the IKKβ/p65-NF-κB signaling pathway in human platelets is greatly amplified in response to thrombin or collagen. Blocking NF-κB activation by BAY11-7082 and Ro106-9920 inhibits platelet aggregation and granule release *via* the blockade of the ERK-cPLA$_2$-TXA$_2$ pathway, fibrinogen binding, platelet adhesion and, spreading in activated platelets [67]. Accordingly, suppressing NF-κB activation may be a potential target for inhibiting platelet aggregation. Previous studies have confirmed that the anti-inflammatory effect of PPAR-γ is associated with reducing NF-κB activation resulting from inhibiting IKKs in activated macrophages [68]. Therefore, the NF-κB activation is also regulated by PPARs. However, the effect of PPAR-γ on NF-κB activation is cell type and PPAR isoform specific [69].

9. Antiplatelet activity of nifedipine is mediated by NF-κB activation *via* PPAR-β/-γ-dependent manner

Treatment with nifedipine decreass NF-κB activation by inhibiting IKK-β/IkBα phosphorylation in collagen-stimulated platelets. The inhibition of NF-κB activation is significantly reversed by specific PPAR-β/-γ antagonists, supporting the notion that PPAR-β/-γ negatively regulates NF-κB activation in platelets. Additionally, activation of NF-κB with betulinic acid (BetA) abolishes the nifedipine's inhibition of intracellular Ca^{2+} mobilization and platelet aggregation, indicating that PPAR-β/-γ-mediated NF-κB activation involves the antiplatelet activity of nifedipine [70]. Notably, our research demonstrated for the first time that in activated platelets, nifedipine also induces an interaction of PPAR-β/-γ with NF-κB leading to decreased p65NF-κB phosphorylation in the complex. These results may provide a novel mechanism by which PPAR-β/-γ suppresses NF-κB activation in platelets through a direct interaction with NF-κB. Furthermore, the suppression of NF-κB activation by nifedipine is at least partly attributed to PPAR-β/-γ-dependent up-regulation of the NO/cyclic GMP/PKG1 pathway (Figure 3).

The binding of fibrinogen to the surface GPIIb/IIIa complex is a critical final step for platelet aggregation by crosslinking platelets and by the stabilization of aggregates. It has been reported that inhibiting NF-κB activation by NF-κB inhibitors reduces the outside-in/inside-out signaling of GPIIb/IIIa and fibrinogen binding in activated platelets [67]. Furthermore, GPIIb/IIIa is also required for NF-κB activation in human neutrophils [71], suggesting that there is a mutual activation between NF-κB and GPIIb/IIIa. Thus, the decreased surface GPIIb/IIIa expression by nifedipine may be a consequence of PPAR-β/-γ-down-regulated NF-κB activation as evidenced by blocking PPAR-β/-γ activity or enhancing NF-κB activation resulting in an elevated expression of GPIIb/IIIa.

In conclusion, nifedipine significantly increases the activity and intracellular levels of PPAR-β/-γ in activated platelets, which subsequently up-regulates the PI$_3$K/Akt/eNOS/NO/cyclic GMP/PKG cascade leading to the suppression of intracellular calcium mobilization, surface GPIIb/IIIa expression, and PKC activity *via* association of PPAR-β/γ with PKC. In addition, nifedipine is capable of inhibiting NF-κB activity through direct interaction of PPAR-β/-γ with NF-κB. These effects of nifedipine ultimately inhibit platelet aggregation and thrombosis formation. All told, nifedipine may be a potential drug for alleviating atherothrombosis and vascular diseases by targeting PPAR-β/γ-dependent signaling pathways in platelets.

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Review article

Mass spectrometry-based proteomics in Chest Medicine, Gerontology, and Nephrology: subgroups omics for personalized medicine

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ABSTRACT

Mass spectrometry (MS) is currently the most promising tool for studying proteomics to investigate large-scale proteins in a specific proteome. Emerging MS-based proteomics is widely applied to decipher complex proteome for discovering potential biomarkers. Given its growing usage in clinical medicine for biomarker discovery to predict, diagnose and confer prognosis, MS-based proteomics can benefit study of personalized medicine. In this review we introduce some fundamental MS theory and MS-based quantitative proteomic approaches as well as several representative clinical MS-based proteomics issues in Chest Medicine, Gerontology, and Nephrology.

1. Mass spectrometry and proteomics

Proteomics (large-scale analysis of proteins) can directly reflect and characterize the biological function, pathways, activities and subcellular distributions, and thus is most promising and applicable in biomedicine [1]. Mass spectrometry (MS) has become a mainstream and dominant analytic tool for studying proteomics due to high sensitivity, specificity and high throughput in protein characterization including posttranslational modifications [2, 3]. Given powerful technology to decipher biological processes, ever more investigators apply MS-based proteomics to clinical research. This review provides an uncomplicated but broad overview of background and issues in MS-based proteomics: protein digestion, instrumentation, ionization methods, database search, quantitative proteomics. We also discuss MS-based proteomic strategy applied in Chest Medicine, Gerontology, and Nephrology.

1.1. Sample preparation: gel- and solution-based digestion

For identification, proteins can be analyzed with intact form for top-down analysis or enzymatically into peptides for bottom up analysis. Since MS techniques are more sensitive for peptides than for proteins, most proteomic applications adopt bottom-up analysis; enzymatic (such as trypsin) digestion, is widely used to digest proteins into peptides in gels or in solution prior to MS analysis. Gel-based digestion is often used when complex proteins are separated on one- or two-dimensional gel electrophoresis. After separation, proteins trapped in gel spots are excised, washed, then digested with trypsin *in situ*. Digested peptides were often extracted from gel pieces with sequential extraction of 0.1% formic acid (FA), 50%ACN/0.1%FA and pure ACN. Because urea, detergents (SDS, Triton X-100) and salts greatly reduce analyte signals in ESI-MS and MALDI-MS while impairing LC separation, removal of contaminants is a key step in sample preparation [4]. One advantage of gel-based digestion: surfactants and salt contaminants are expunged from gels by washing steps without significant protein loss [5, 6]. Practical gel-assisted digestion for surfactant-enriched protein sample preparation starkly increased membrane proteome recovery [7]. Still, digested peptide recovery of gel-based digestion is often limited by lower extraction

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efficiency of trapped peptides from gel spots; excised gel spots must be cut into smaller pieces for better digestion and extraction efficiency.

In solution-base digestion, urea, detergent or heat is usually added to denature protein for efficient enzymatic digestion. Without trapping proteins in gel, solution-based digestion benefits from higher peptide recovery. However, salts, urea and detergents for digestion must be removed by solid phase extraction (C18 stationary phase) before MS analysis. Recently, a simple universal sample preparation by a filter-aided method developed by M. Mann [8] allowed researchers to use higher amount of detergent or urea for comprehensive proteome analysis. Its lone drawback is longer processing time in multiple centrifugations. Trypsin is most commonly used, owing to high cleavage efficiency and specificity in targeting arginine and lysine at C-terminal. Tryptic peptides are primarily of ideal size and multiply charged suited for identification by tandem mass spectrometry (MS/MS) [9, 10]. In analyzing complex proteome, additional enzyme of endoproteinase Lys-C can be used with trypsin to boost protein digestion efficiency by eliminating the majority of missed cleavages.

2. Ionization methods of ESI and MALDI

ESI and MALDI are two chief ionization methods for charging and transforming proteins/peptides into gas phase available for MS analysis [11]. ESI, meaning dissipate liquid sample homogeneously, was not applied to analysis of large molecules until 1988. John Fenn *et al.* demonstrated its capacity for analyzing large biomolecules [12]. By applying positive or negative direct-current (DC) voltage (+2~4 kV or -2~4 kV) at an electrically conducted spray tip, sample solution is dispersed by electrospray into a fine aerosol. Sprayed fine aerosol were charged and continuously evaporated based on ion evaporation model and charge residue model, which allow analyte charged in gas phase and transferred into MS analyzer [13]. When operating flow rate is above the optimal spray flow rate of the ESI tip orifice, ESI ion signals increase linearly with analyte concentrations until it saturates in MS analyzer system [14]. For more sensitive ESI-MS analysis, Wilm and Mann have introduced nanoelectrospray (nanoESI) technique [15] that uses extremely small needle orifices (nanospray tips with 20 μm orifice iscommercially available) for spray flow rate below 1 μl/min. Initial created smaller droplets enable establishment of high surface-volume ratio of droplets, early fissions without extensive evaporation, thus increasing sampling efficiency and tolerating higher salt contamination. Since nanoESI is operated in nanoliter flow rate, nanoESI is broadly coupled to nanoLC (LC flow rate: 200-400 nl/min) for more sensitive analysis in proteomics [16].

MALDI is a technique involving serial energy transfer and ionization processes. Samples are first mixed with MALDI matrix (i.e. α-Cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), Sinapinic Acid (SA)) on a spot of a MALDI plate. After air-dry and cocrystallization, sample and MALDI matrix are colocolized in crystals. With laser beam irradiation on the crystals, MALDI matrix absorbs laser energy and help analytes desorb from crystals into gas phase [17]. Homogenous crystalscan be observed by video camera set up in MALDI ion source and can provide better signal reproducibility and sensitivity. When applying matrix on samples, the ratio of matrix and analyte sometimes should be optimized for better sensitivity. In addition, thicker crystals significantly reduce peak resolution. Because sample spot homogeneity is the major concern to influence signal reproducibility in MALDI, hydrophobic MALDI target has improved sample homogeneity as well as concentrate analytes [18]. MALDI has been broadly used in analyzing small molecules, polymer, peptides, proteins, oligonucleotide sequencing, and DNA [19]. Compared with ESI ionization method, MALDI has advantages of rapid sample preparation, and more tolerance of salts and detergents. However, because MALDI is usually suffered from poor reproducibility in absolute signal intensity from sample well-to-sample well, MALDI is not commonly used in absolute quantitative approach unless an internal control signal was introduced [20].

3. Basic description of mass analyzer

In the growing field of proteomics, some major types of mass analyzers are frequently used, such as triple quadrupole, ion trap, orbitrap, fourier transform ion cyclotron resonance (FT-ICR) and TOF instruments [21]. Each analyzer has its superiority and limitations in performance: e.g., intra-spectrum dynamic range (the range over which the ion signal is linearly proportional to the analyte concentration), sensitivity, mass range, scan speed, scanned duty cycle, accuracy, and resolving power (the ability to differentiate two adjacent peaks). These analyzers can operate alone or couple in series, named hybrid mass spectrometer: e.g., quadrupole-TOF, quadrupole-orbitrap, ion trap-orbitrap, ion trap-TOF, ion trap-FTICR etc., to provide a better performance by merging the strengths of each [22].

In quadrupole-MS, ion mass scan is carried out by creating time-varying electric fields constructed by DC and RF voltage on four hyperbolic rods positioned symmetrically along one axis. Potential of DC applied to adjacent rods are opposite to each other. Combined DC and RF voltage can then create a stability potential diagram for a given ion mass stably pass through the quadruple and be detected [23]. Thus, quadrupole can act as a mass filter for ion mass scan by varying the RF and DC voltages or as an ion guide for ion transmission ion by setting RF voltage only. In tandem MS of triple quadrupoles, the first quadrupole (Q1) act as a mass filter for ion scan or ion selection, the second quadrupole (Q2) act as ion guide with RF only mode for collision induced dissociation of ions, which were then scanned by the third quadrupole (Q3). Figure 1 shows different scanning modes by MS/MS. This tandem MS (MS/MS) in space includes precursor ion scan, product ion scan, neutral loss scan, selected ion monitoring (SRM), and multiple reaction monitoring (MRM), which can greatly reduce chemical noises to improve sensitivity. MRM, a scan mode of multiple SRM transitions within the same MS analysis, detects precursor/fragment ion pairs. Due to superior sensitivity of MRM function, nanoLC-ESI triple quadruple have been developed for biomarker validation in large sample size in target proteomics instead of ELISA and Western blot [24, 25].

Similar to quadrupole-MS, the operating principle of ion trap is based on electric fields constructed by a ring (RF voltage) and two end caps (alternating current (AC) voltage), which create a stable potential diagram for storage a given mass ion in an ion trap. For scanning ions of an ion trap, ions are detected after they exited the end cap electrode by ramping RF voltage on ring electrode or by causing resonant ejection on end cap electrodes [26]. Advantages of ion trap analyzer include fast scan speed, MS^n ability (e.g. MS, MS/MS and MS/MS/MS), and high sensitivity [27].

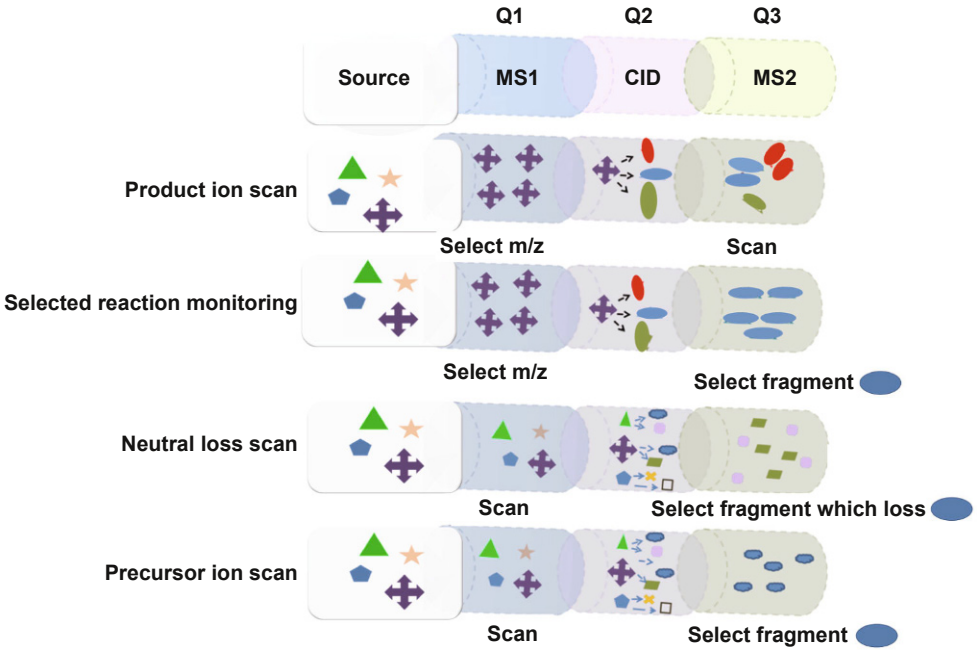


Fig. 1 - Scan modes of tandem mass spectrometry. (1) Product ion scan: select Q1 precursor ion and scan Q3 production. (2) Selected ion monitoring: select precursor ion in Q1 and monitor one or more fragment ions in Q3. (3) Neutral loss scan: scan all ions in Q1 and select ions with neutral loss in Q3. (4) Precursor ion scan: scan precursor ion in Q1 and select certain fragment ion in Q3, all collision induced dissociation carried out in Q2.

Ion trap can perform MS/MS in time with product ion scan and MRM. Yet when overloading ions in the ion trap, space-charge effect will result in poor peak resolution and mass-shift. Therefore, most ion trap equipped with pre-analysis function (automatic gain control, AGC) to estimate proper ion loading time [28]. The geometries of electrotrodes have revolutionized from three-dimensional to linear ion trap to upgrade efficiency and capacity, sensitivity, detection dynamic range, and scan rate [29].

In TOF analyzer, mass-to-charge (m/z) ratio of each ion is determined by flight time of charged ions over a vacuum tube of specified length inversely proportional to [30]. Because TOF can record all ions simultaneously and separate ion based on each m/z ratio, it is superior of fast scan speed. However, the resolution and mass accuracy of TOF is dampened by several factors including sample thickness difference, initial ion velocities difference, and turn around effects, etc. [31]. Design of delay extraction and reflectron have greatly improved resolving power, mass accuracy, and prompt TOF as high-resolution mass analyzer [32]. Nowadays TOF is frequently coupled with another TOF (such as MALDI TOF-TOF) or quadruple (Q-TOF) for high-quality MS/MS spectrum. Owing to feasibility with LC-ESI system, nanoLC-ESI-Q-TOF has been widely used in bottom-up proteomics for high resolution, high scan speed, and high mass accuracy [33].

In FT-ICR-MS, with applying a homogenous unidirectional magnetic field, ions of specific m/z will undergo cyclotron motion with corresponding frequency characteristic of their m/z ratio after excited by resonant rf electric field [34]. All ions of the same m/z travel in a spatially coherent packet. Each ion packet induces current on a pair of opposed electrodes to yield time-domain signal then deconvoluted by Fourier transformation to obtain their corresponded m/z [35]. Among current mass analyzers, FT-ICR affords highest mass resolving power (~1,000,000 at FWHM) and mass accuracy (<1 ppm) [36]. High accuracy of measuring mass of analyte ion can help to determine its accurate elemental composition. In proteomics, high resolution power of FT-ICR has superiority in identifying protein either using "bottom-up" or "top-down" approach [37, 38]. Still, with scan speed of FT-ICR MS slower, FT-ICR MS is not widely used in quantitative proteomics, yielding less peptide fragmented ion spectra in complex proteome samples [37].

Orbitrap-MS can be viewed as a modified form of Kingdom trap or modified form of ion trap. The difference between orbitrap and ion trap is that the field of orbitrap is electrostatic while the field of quadrupole ion trap is electrodynamic [29]. Advantages of orbitrap include high mass accuracy, and less space-charge effects thus wilder dynamic range and higher high mass/charge range [29]. However, because the mass signals were based on the imaging current, orbitrap is still limited by its slower scan speed compare to ion trap and TOF systems. Linear ion trap triple quadruple (LTQ) and quadruple have been both successfully hybrid to orbitrap by insertion of a c-trap, which can storage ions and reduce kinetic energy of ions from ion source and then injected ions into orbitrap for analysis. In initial development of LTQ-orbitrap design, orbitrap was used for precursor ion scan (MS scan) to obtain accurate ion mass with high resolution, and LTQ was used for product ion scan (MS/MS scan) to obtain abundant MS/MS spectra with high throughput. Parallel scans can dissolve slow scan rate problem of orbitrap in proteomics [39]. Recently, higher energy collision dissociation (HCD) cell was adjacent to orbitrap for performing quadruple-like MS/MS without losing low mass ions. This improved MS/MS function allows LTQ orbitrap-MS applicable to iTRAQ quantitative proteomics, in which low mass ion tags (114, 115, 116, 117 m/z) in MS/MS spectra were used for

quantitation. More recently, high field of orbitrap has improved scan speed to 18 Hz at resolution setting of 15,000 at 200 m/z. (Q Exactive HF, Thermo).

4. Database searching and protein identification

MS-based approaches are current popular methods for protein identification based on well-established genomic and protein databases as well asbioinformatics tools. Higher mass accuracy and resolution of MS data can provide more confidence protein identifications. The "peptide mass fingerprinting" (PMF) method is the fastest method to identify proteins recovered from 2DE-based proteomics. In PMF, proteins are first digested, then detected by MS full scan to obtain peptide signals as many as possible. Detected peptide signals are compared with theoretically expected peptide masses in a protein database. A score was used to describe results of each comparison [40]. For protein identification with MS/MS spectra, *de novo* sequencing and "peptide fragment fingerprinting" are widely used. *De novo* methods are used to identify proteins when genomes are not known and utilize computational approach to deduce (partial) sequence of peptides directly from experimental MS/MS spectra [41]. Peptide fragment fingerprint entails comparing experimental MS/MS spectra against those theoretically generated peptide candidates [42]. There are numbers of searching algorithms for protein identification: e.g, probability-based scoring in MASCOT, cross correlation scoring in SEQUEST, and hypergenomic scoring in X!TEM [43-45]. Searching algorithms can only identify proteins with sequences in database, while bottom-up method has limitations: e.g., unanticipated tryptic cleavage by-products, limited identification rates of LC-MS/MS runs [46].

4.1. Quantitative proteomics

MS-based quantitation has been a major wok in proteomic research [47]. The current available quantitative methods divide into gel-based and gel-free nano LC-MS/MS quantitative proteomics [48, 49]. In gel-based proteomics, complicated protein mixture was analyzed by two-dimensional gel electrophoresis (2DE). Protein mixture was first separated on an immobilized pH gradient strip according to isoelectical points of proteins. Then, the strip was put on the top of SDS-PAGE for the second dimensional separation according to their molecular weight. 2DE presents a quantitative map of proteome, providing information about the estimated pI and molecular weight of proteins, the levels of protein expression, and post-translation modification [6]. Each individual sample or pooled sample group is performed on each gel. Replicated runs should be performed to reduce quantitative errors. In quantitation, 2D-gels of different sample groups were recorded by image software, which can calibrate spot location and intensity, and output relative quantitative information.

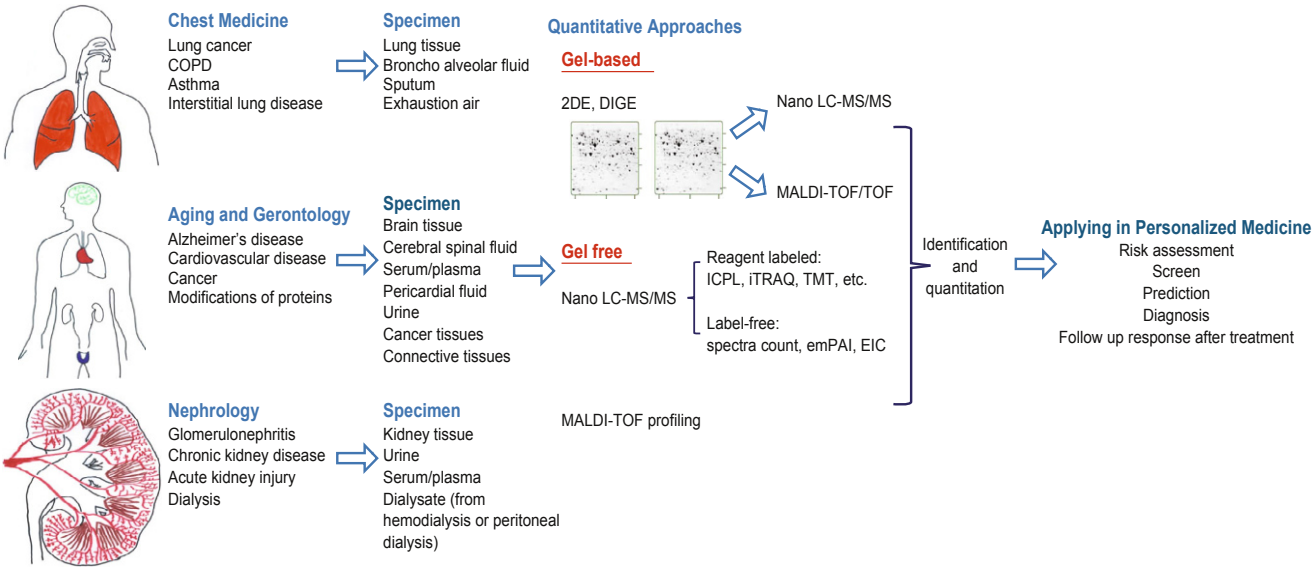
Gel-based approaches have several important advantages for complex protein mixtures: high resolution for separating complex proteins, visualized post-translational modifications and removable salts/detergents in gel-based protein digestion procedure [50, 51]. However, the well-recognized limitations of 2DE include low reproducibility, and inability of analyzing membrane proteins. To improve reproducibility and accuracy in quantitation of 2DE, difference gel electrophoresis (DIGE) approach was introduced by the principle of fluorescence pre-labelling sample proteins before 2D electrophoresis. Proteins of different sample group are separately labeled with different fluorescence reagents (cyanine dyes with different excitation and emission wavelengths) and then pooled into a sample mixture, followed by 2D electrophoresis analysis on a single gel. The different extracted proteins can be visualized under corresponding excitable wavelength and then enable the comparative quantitation among these proteins [48]. However, the colorless DIGE gels should be stained with Coomassie blue or silver stain and carefully recalibrated of spot position before excising interested protein spot for MS analysis.

The other major quantitative proteomic strategy is the gel-free approach using nano LC-MS/MS which can be further divided into reagent labeled and label free approaches.

The label-based technologies are based on the principle that labelled peptides in different sample groups with a combination of non-radiative isotopes (e.g. C^{13}, H^{2}, N^{15}). The different sample groups labeled with different tags were then mixed into the same sample solution prior to nano LC-MS/MS analysis. In nano LC-MS/MS analysis, peptides of same amino sequence from different sample group labeled with different mass tags exhibit the same chromatographic and ionization properties but can be distinguished from each other by a mass-shift signature in MS spectra or mass tags in MS/MS spectra [52]. The label-based technologies include chemical labeling methods of isotope-coded affinity tags (ICAT), isotope-coded protein labelling (ICPL), isobaric tags for relative and absolute quantification (iTRAQ), tandem mass tag (TMT), metabolic labeling SILAC, and $^{14}N/^{15}N$ Labelling [53-56]. These labeling techniques contain well-designed isotope reagents to label peptides (ICPL, ICAT, iTRAQ, TMT) or proteins (ICPL, SILAC) for comparing protein expression changes in different biological samples.

Compared with label-based quantitation approaches, label free quantitation has the advantages of lessening the time and complexity of multi-step labeling process, minimizing the sample loss, and the cost of reagents [57]. In label-free methods, each sample or pooled sample group should be separately analyzed without mixing with other sample group. There are two kinds of label-free quantitation approaches based on spectra counting and ion intensity. Spectra counting, defined as by comparing the number of identified MS/MS spectra, is a semi-quantitative approach providing a low cost and rapid evaluation of protein expression difference. Liu *et al.* have observed a strong linear correlation between MS/MS spectra counts and relative protein abundance [58]. Many strategies and statistical tools have been developed for analyzing spectral count data and reducing the variations from replicated runs [59-62]. emPAI (exponentially modified PAI) has been developed to estimate the abundance of proteins [59], which has been incorporated into MASCOT (a commonly-used protein searching engine) for rapid evaluation of protein abundance. However, spectra counting results are only acceptable for samples with relative large quantitative differences and for proteins having the numbered of identified peptides exceeding a certain threshold [63].

Label-free quantification approaches based on ion peak intensities by extracting ion chromatogram (EIC) from MS spectra is more acceptable due to its better quantitation results. In each nanoLC-MS run, the intensity and elution time of each peptide ions was processed, recoded as a quantitative "molecular feature" and form a feature map. These feature ions of different feature maps acquired from different nano LC-MS runs are aligned according to their accurate masses and reproducible LC retention time. Comparison of feature abundances on different maps (representing nano LC-MS runs of different samples) reveals relative



2DE: 2-Dimension Electrophoresis; DIGE: Difference Gel Electrophoresis; ICPL: Isotope-coded protein label, iTRAQ: Isobaric tags for relative and absolute quantitation; emPAI: Exponentially modified protein abundance index; EIC: extracted ion chromatography

Fig. 2 - Concept and current progress of mass spectrometry-based proteomics in Chest Medicine, Aging and Gerontology, and Nephrology.

changes between peptide amounts [64]. Peptide ions with relative fold changes were then integrated to its MS/MS spectra and protein database search to obtain the protein sequence. Fold changes of all peptides from the same protein are averaged to obtain relative expression level. More concepts and tools for label-free peptide quantification has been recently reviewed by Nahnsen S. *et al*. [65].

In addition to nano LC-MS/MS approaches, MALDI has also been used as an relative quantitative tool to rapidly discover biomarkers in bacterial [66], serum, urea and saliva. With coating different stationary phases (C18, C8, ion exchange, etc.) on magnetic particles or on MALDI plate (surface-enhanced laser desorption/ionization (SELDI), *et al*. [67]) for specific biomolecular purification, these sample preparation methods can simplify sample complexity and therefore enhance detection sensitivity of certain species. In addition, SELDI-TOF or MALDI-TOF with magnetic particle purification approaches in protein profiling can detect expression changes of protein isoforms. However, these MALDI-TOF based protein profiling methods are still restricted by poor sensitivity in detecting larger proteins (>20 kDa) and ion suppression effects which results in limited peak ions in complex samples.

5. MS-based proteomics in Chest Medicine, Gerontology, and Nephrology

The field of MS-based proteomics has getting matured, being able to analyze the complex proteome with consistency, and even to explore proteome dynamics [68]. For years, the gap and transition between discovery science and clinical medicine has been wide and slow. However, the potential for MS-based proteomics, applied as a methodology in clinical practice, is promisingly powerful to bridge the gap and accelerate the transition. Here, we describe applications of MS-based proteomics in Chest Medicine, Gerontology and Nephrology, and the general approaches and workflow was shown in Figure 2.

5.1. Chest Medicine

The majorities of proteomic studies in Chest Medicine have been straightforward focusing on major diseases: e.g., lung cancer, obstructive airway diseases like chronic obstructive pulmonary disease (COPD) and asthma. Some MS-based proteomics studies of lung cancer, COPD and asthma were summarized in Table 1 [69-81]. Lung cancer is the cancer leading high mortality worldwide [82]. The delayed diagnosis at last advanced stage of lung cancer accounts for its high cancer-related death rate. Several studies have identified certain mutation of susceptible genes to lung cancer, including epidermal growth factor receptor (EGFR) gene and nucleotide excision repair genes [83, 84]. Smoking, radon, second-hand tobacco smoke, and other indoor air pollutant are well-recognized environmental carcinogens of lung cancer [85]. Since the pathogenesis of lung cancer involves the complex interaction of host genetic predisposition and environment, it is unlikely to diagnose lung cancer based on the incomplete picture provided by gene profiling and exposed environmental factors of individuals [86]. Several screening tools as sputum cytology, interval chest x-rays, and computed tomography scans in smokers have proven cost-ineffective in reducing lung cancer mortality rates [87].

MS-based proteomics strategy has shown potential in finding out the biomarkers of lung cancers from several perspectives inclusive of proteome of lung cancer tissue, serum, saliva, braonchoalveolar fluid, and exhaustive air [69-73]. Protein profiles of tissue can distinguish lung tumor from normal tissues, separate malignancy from pre-malignant pulmonary epithelium, and predict the prognosis of lung cancer patients [69-71]. Non-invasive approaches including analyzing proteins profiling of saliva and exhaled breath condensate have been promising in detecting lung cancer with AUC up to 0.90 [72, 73]. MS-based proteomic

Table 1 – Selected representative studies of Mass spectrometry-based proteomics in Chest Medicine.

Disease	Specimen	Marker	Proteomic technique	Ref
Lung cancer	Tissues	Proteins profiling	MALDI-TOF	[69]
	airway epithelium	Proteins profiling	MALDI-TOF	[70]
	Saliva	Calprotectin, annexin A1, haptoglobin hp2, α 2-glycoprotein	2D-MS	[72]
	Tissues	protein profiling, 17250 Da (-)	SELDI-TOF	[71]
Lung cancer treatment response	Serum	Predictive algorithm	MALDI-TOF	[74]
COPD	Tissues	matrix metalloproteinase -13 and thioredoxin-like 2	MALDI-TOF	[75]
	bronchoalveolar lavage fluids	neutrophil defensins 1 and 2, S100A8 (calgranulin A), and S100A9 (calgranulin B)	SELDI-TOF	[76]
	Sputum	203 distinct proteins, protein profilings	CapLC-Q/TOF-MS	[77]
	Sputum	polymeric immunoglobulin receptor	2D-DIGE-MS	[78]
Asthma	exhaustion air	Leukotrienes (LT) D4, LTE(4), LTB(4)	GC-MS	[79]
	exhaled breath	LTB(4)	LC-MS	[80]
	Urine	LTE4	LC-MS	[81]

MALDI-TOF: matrix-assisted laser desorption inoization-time of flight; SELDI: Surface-enhanced laser desorption/ionization; 2D: two dimensional gel electrophoresis; DIGE: difference gel electrophoresis; LC: liquid chromatography; GC: gas chromatography; Q: quadrupole.

strategy is not only used in diagnosing lung cancer, further it can also be used in predicting the response to target therapy for lung cancer. For the majority of patients with advanced lung cancer, the most important biosignature is in predicting response to target treatment to achieve the goal of "personalized therapy". Taguchi *et al*. developed MALDI MS algorithm to predict prognosis of non-small cell lung cancer patients after treatment with epidermal growth factor receptor tyrosine kinase inhibitors, which may help in the pretreatment selection of appropriate subgroups of lung cancer patients [74]. Although the results seem promising, these proteomic strategies remain investigational and await future validation of the application in screen, diagnosis, and pre-treatment selection of patients of lung cancer before they can be carried out in clinical practice.

5.2. Aging and Gerontology

The study of elderly people whose age is more than 65 years is termed as geriatrics. In addition to the chronological definition, aging could still be defined biologically, physically and mentally. Biological aging represents a fundamental process that has a higher risk in the development of cancer, neurodegenerative, and cardiovascular diseases (CAD) than non-elderly [88].

With increasing longevity and decreased fertility rate, the elderly population is getting steadily increased worldwide. Age-related chronic diseases, termed comorbidity and multimorbidity started to catch clinicians' attentions [89]. CAD, Alzheimer's disease, and cancer can be considered as accumulating disease predominantly observed in the aging period. Proteomics approach can reveal the phenotype of aging and may provide an insight for investigating the mechanism of these chronic diseases. Some important studies related to aging disease are listed in Table 2 [90-108].

MS can examine chemical structure and organizing process of amyloid beta-protein from Alzheimer's brain [95, 109]. In addition to apply MS-based techniques in probing etiology and mechanism of Alzheimer's disease, more studies have adopted MS-based proteomics for biomarker discovery of Alzheimer's disease. Cerebral spinal fluid and serum have been the material for MS-based non-target proteomics and target-proteomics for discovering and validating biomarkers of Alzheimer's disease, respectively [96, 97]. Likewise, MS-based proteomics lent insight into the pathogenic role of deregulated protein in pathophysiology of Alzheimer's disease, which is helpful as a treatment target for drug discovery [97-99, 110]. In addition to effects of aging on developing disease, it is also observed that age had similar detrimental influence on proteins. Age-related modification (phosphorylation, oxidation, glycation, racemization, nitration, etc.) are also observed and may induce disease [106, 107].

5.3. Nephrology

Some MS-based proteomic studies encompassing ischemic acute kidney injury, contrast nephropathy, urolithiasis, kidney rejection, and lupus nephritis were also listed in Table 3 [69, 111-130]. The gold standard of diagnosing glomerulonephritis (GN) is renal biopsy, which is invasive and risky. MS-based proteomic studies have uncovered new biomarkers and pathophysiology of GN. Beck *et al*. have used MS approaches for renal tissue specimen analysis from patients with idiopathic membrane nephropathy. to identify M-type phospholipase A2 receptor, as a potential marker that differentiates patient groups between idiopathic membrane nephropathy and other GN [122]. A pattern consisting of 22 polypeptides from a capillary electrophoresis-mass spectrometry (CE-MS) study has successfully distinguished IgA nephropathy from healthy controls, diabetic nephropathy, minimal change disease, and focal segmental glomerulosclerosis with 100% sensitivity [131]. There have been proteomic studies on peritoneal dialysate from patients receiving peritoneal dialysis [132]. It is believed that proteomics of peritoneal dialysate can enhance understanding of peritoneal dialysis and lend potential biomarkers for predicting peritoneal damage [128].

5.4. Omics-based personalized medicine: an evolving art of clinical practice

The revolution of medicine has entered a new era, with major

Table 2 – Selected representative studies of mass spectrometry-based proteomics in Aging and Gerontology.

Disease	Specimen	Marker	Proteomic technique	Ref
CAD	Plaque	SDF1-α, unprocessed TGF-β1, basic FGF, PDGF	LC-MS	[92]
	atherosclerotic plaques	Protein expression map	MALDI-TOF	[90]
	Platelet	Secretogranin III, cyclophilin A, and calumenin	2D-MALDI-TOF	[93]
	Blood	vimentin, mannose binding lectin receptor protein, S100A8 calcium-binding protein	2D-MALDI-TOF	[140]
Alzheimer's disease	cerebral Cortex	amyloid ß-protein	HPLC-MS	[95]
	cerebrospinal fluid	unknown 7.7 kDa polypeptide, 4.8 kDa VGF polypeptide, cystatin C, two beta-2-microglobulin	SELDI-TOF	[96]
	Serum	plasma ApoE levels had no obvious clinical significance	HPLC-QTRAP	[97]
	Leukocyte	14-3-3 protein epsilon and peroxiredoxin 2; and eight down-regulated proteins, actin-interacting protein, mitogen activated protein kinase 1, beta actin, annexin A1, glyceraldehyde 3-phosphate dehydrogenase, transforming protein RhoA, acidic leucine-rich nuclear phosphoprotein 32 family member B,	MALDI	[99]
Cancer	Urine from prostate cancer	Polypeptides	CE-MS	[102]
	Urine from urothelial cancer	Polypeptides pattern	CE-MS	[141]
PTM	Lens	N-terminal racemization.	LC-MS/MS	[107]

PDGF: pigment epithelium-derived factor; VGF: vessel growth factor; SDF1-α: Stromal cell-derived factor α; TGF-β:Transforming growth factor-β1; CE-MS: capillary-electrophoresis-coupled mass spectrometry; PTM: posttranslational modification

Table 3 – Selected representative studies of mass spectrometry-based proteomics in Nephrology.

Disease	Specimen	Marker	Proteomic technique	Ref
AKI	Urine	IP-10	SELDI-TOF	[115]
	Urine	Angiotensinogen	2-D LC-MS/MS	[116]
	Urine	Protein profiling	SELDI-TOF	[118]
GN	Urine	isoforms of hepcidin, fragments of alpha1-antitrypsin and albumin	SELDI-TOF	[119]
	kidney tissues	C3α and C3ß	LC-MS	[120]
	kidney tissues	Ig heavy chain amyloid.	LMD/MS	[121]
	serum	M-Type Phospholipase A2 Receptor	LC-MS	[122]
CKD	Urine	Peptide profiling	CE-MS	[125]
	Urine	urinary proteome-based classifier (CKD273)	CE-MS	[126]
	Urine	12-peak proteomic signature	SELDI-TOF	[127]
Dialysis	Peritoneal dialysate	Protein profiling	nano LC-MS/MS	[129]
	Dialysate	Protein profiling	SELDI-TOF	[130]
	Dialysate	Protein profiling	nano-UPLC-MS/MS	[132]

AKI: acute kidney injury; GN: glomerulonephritis; IP-10: interferon-inducible protein-10; LC: liquid chromatography; LMD: laser microdissection; CE-MS: capillary-electrophoresis-coupled mass spectrometry.

achievements in recent decades. The challenging progress is the eager to pursue personalized medicine. Personalized medicine, meaning to take into consideration the whole system biologic status of an individual enables the public health scientists and clinicians to choose and tailor the appropriate screening strategy, intervention, drugs to fit the need of biological variability of each individual as possible [133]. Certainly, considering the heterogeneity of genome, epigenome, and the resulting associated phenotype, it is unlikely and cost to design a specific examination or create a medication just unique to one patient. American officials have defined personalized medicine with greater precision as "ability to classify individuals into subpopulations that differ in their susceptibility to a particular disease or their response to a specific treatment" [134].

In the past, universal personalized medicine seems impossible to carry out either in Western or Chinese clinical practice. In Western medicine, what most time physician spent in clinical practice is disease recognition and decision making. Physicians are trained to cure disease regardless of biological variance among individuals. Conceptually different, traditional Chinese medicine considered ill individual as a whole, thought of system medicine, and aimed to achieve system balance based on the con-

cepts of yin–yang, Qi and Blood, and Zang-fu organ [135, 136]. However, traditional Chinese medicine could not be carried out and quantified uniformly by each practitioner, since the practice of Chinese medicine depends largely on imagery, intuitional, and holistic thinking [137].

Completion of Human genome project allows illumination of the human genome and eager in maturing of personalized medicine to resolve irreconcilable differences of philosophies between Western medicine and Chinese medicine [138]. Despite the availability of complete genome sequence, researchers cannot predict manifestation of diseases of physiological process very precisely, given expression of organism activity is much closer to level of functional genome rather than that of genome. Awareness of dynamic complexity of biological activity within human body lead personalized medicine moving beyond genomics, epigenomics, transcriptomics, and finally proteomics to get direct levels of functional insight. Chen *et al.* studied the proteome of individual colorectal cancer tissues of each patient and used it to establish a pilot model of MS-based proteomics in personalized medicine [139]. This study offers a roadmap for future related studies of personalized medicine; MS-based proteomics of personalized medicine, a key strategy to reform healthcare, is still in its infancy. Issues in clinical aspects of personalized medicine merit attention: well-controlled study for subgrouping; cut-off value and threshold of biomarkers for disease detection and treatment response variant among persons; effects of environment, genetics, and disease variability in a population. Proteotype within the organism is dynamic and varies with time. How to summarize and signify results of this dynamic proteome across samples and individuals poses a challenge in near future.

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Original article

Lyophilized particles and ethanolic extracts of *Antrodia cinnamomea* mycelia suppress the tumorigenicity of head and neck cancer cells *in vivo*

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ABSTRACT

Head and neck cancer (HNC) is one of the most common forms of cancer in Taiwan. In addition, head and neck cancer cells (HNCs) are highly tumorigenic and resistant to conventional therapy. Therefore, development of new therapeutic regimens that are adjuvant to conventional treatments would benefit future head and neck cancer therapy. In this study, we found that the lyophilized particles and ethanolic extracts of *Antrodia cinnamomea* mycelia inhibited the tumor growth of HNCs by xenograft assay *in vivo*. Moreover, administration of lyophilized particles or ethanolic extracts to nude mice did not cause significant side effects. Our study revealed that the *Antrodia cinnamomea* mycelia extract (ACME) efficiently inhibited the tumorigenicity of HNCs without causing organ failure. Furthermore, it showed that ACME may work as a novel drug candidate for alternative treatments for head and neck cancer.

1. Introduction

Head and neck cancer (HNC) represents the sixth most common form of cancer with an estimated 600,000 new cases annually worldwide [1]. Head and neck squamous cell carcinoma (HNSCC) represents more than 95% of all head and neck cancers [2]. In spite of the many advances in our understanding in prevention and treatment of other types of cancers, the five-year survival rate after diagnosis for HNSCC remains low, at approximately 50% [3]. Due to a high recurrence, a high mortality rate, and a resistance to conventional therapies, the development of new chemopreventive agents for HNSCC that are effective on high risk populations (or patients) and that are adjuvant to conventional treatments is an important research priority.

Antrodia cinnamomea, also called *Antrodia camphorata,* a rare medical mushroom of the family *Polyporaceae*, mainly grows on the inner wood wall of *Cinnamomun kanehiraihay (Lauraceae)* in Taiwan [4]. In traditional Taiwanese medicine, the fruit bodies of *Antrodia cinnamomea* have been widely used to treat diarrhea, intoxication, hypertension, hepatoprotection, itchy skin [5], and cancer prevention [6]. However, the fruit bodies of *Antrodia cinnamomea* are rare and expensive, partially due to the difficulty in cultivation [6]. The submerged culture of *Antrodia cinnamomea* mycelia is one of the most effective methods for application in the formulation of nutraceuticals and functional foods [7]. The biological functions and activities of *Antrodia cinnamomea* mycelia extract (ACME) have been identified [6]. In our study, we found that YMGKI-1, one of the active components from ACME, can inhibit cancer-initiating cell properties through exaggerated autophagic cell death [8]. However, the anticancer effect of the crude ACME in HNSCC with animal models remains unclear.

In the present study, we examined the therapeutic effect of lyophilized particles and ethanolic extracts of *Antrodia cinnamomea* mycelia by xenograft assays. Our data showed that oral feeding with ACME reduced the tumor growth of HNSCC in

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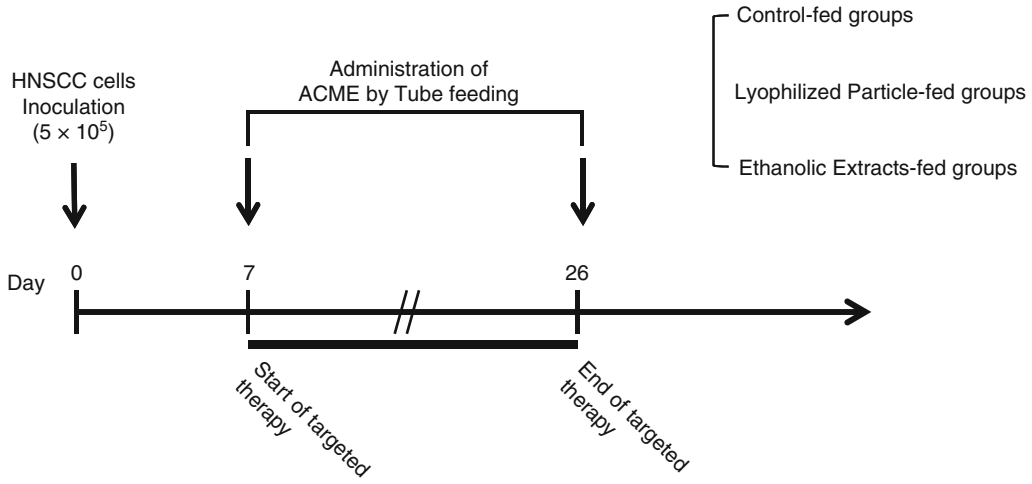

Fig. 1 - Overview of the xenograft mice model with ACM or ACME feeding procedure. Parental HNSCC cells (5×10^5 cells) were subcutaneously implanted into the back of nude mice to develop tumor to a size about 0.1 cm^3. At day 7 after cells implantation, tumor-bearing nude mice were fed with lyophilized particles or ethanolic extracts diet (3 times per week) for 21 day by tube feeding, respectively.

tumor-bearing mice without causing organ failure. Thus, ACME may work as a novel drug candidate for alternative treatments for head and neck cancer.

2. Materials and methods

Preparation of lyophilized particles and ethanolic extracts of *Antrodia cinnamomea* mycelia (ACM) were obtained from the Biotechnology Center, Grape King Inc., in Taoyuan County, Taiwan [7]. Matured mycelia were separated from the red-brown broth and then lyophilized, ground to a powder, and stored at room temperature [9]. Then, the lyophilized particles of *Antrodia cinnamomea* mycelia were used for this study. To prepare the ethanolic extracts of ACM, 1 gram of the above lyophilized particles was further extracted with 95% ethanol at 30°C for 24 h. The filtrates dissolved in 95% ethanol were dried under a vacuum to collect the ethanolic extracts of ACM [10].

2.1 Cell lines

SAS tongue carcinoma cells, human HNSCC cell lines, obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) were cultured in a DMEM medium containing 10% fetal bovine serum (Grand Island, NY) [11]. Cells were cultured at 37°C in a 5% CO_2 environment. Short tandem repeat (STR) genotyping was performed for authentication of used cell lines by Genelabs Life Science Corporation (Taipei, Taiwan).

2.2 *In vivo* tumorigenic assay

All of the animal practices in this study were approved and were in accordance with the Institutional Animal Care and Use Committee (IACUC) of National Yang-Ming University, Taipei, Taiwan (IACUC approval nos. 1001223 and 991235). The antitumorigenic effect of lyophilized particles and ethanolic extracts was examined in 6-week-old nude BALB/c nu/nu mice (n = 4 per group). HNSCC cells (5×10^5 cells) were subcutaneously injected into the back of the nude BALB/c mice (n = 4 per group). Tumors became palpable in about a week. Then, the lyophilized particles or ethanolic extracts were fed by tubing. Treatments were done on a schedule of three times per week for 21 days, after which tumor volumes were determined. The volume of the tumors was calculated *via* the following formula: (Length × $Width^2$)/2 [12].

2.3 Statistics

An unpaired *t*-test was used for statistical analysis. The results were considered to be statistically different when the *P* value was <0.05.

3. Results

3.1 Anti-tumorigenic ability and side effects of ACM and ACME in tumor-bearing nude mice

Antrodia cinnamomea has been used for treatments of diseases and illnesses such as diarrhea, intoxication, hypertension, abdominal pain, itchy skin and some forms of cancer [13]. With this in mind, we wanted to determine if ACM and ACME treatment could attenuate the tumor growth of HNSCC *in vivo*. To investigate whether treatment of lyophilized particles and ethanolic extracts of *Antrodia cinnamomea* could exhibit anti-tumorigenic effects, BALB/c mice were inoculated with SAS cells. When the tumors became palpable, tumor-bearing nude mice were fed with either a lyophilized particles or an ethanolic extracts diet 3 times per week for 21 days by tube feeding, (Figure 1). Effectively, tumor-bearing mice receiving either the lyophilized particles or the ethanolic extracts treatment afterward displayed reduced tumor growth and tumor weight in comparison to that of the control mice (Figure 2A and 2B). As shown in Figure 2C, the mean tumor volume reached 1 cm^3 in the control mice 4 weeks after tumor injection; in contrast, a significant suppression of tumor volume was observed in the mice that were tube fed a diet of either lyophilized particles or ethanolic extracts. The antitumorigenic ability of lyophilized particles was dose-dependent with

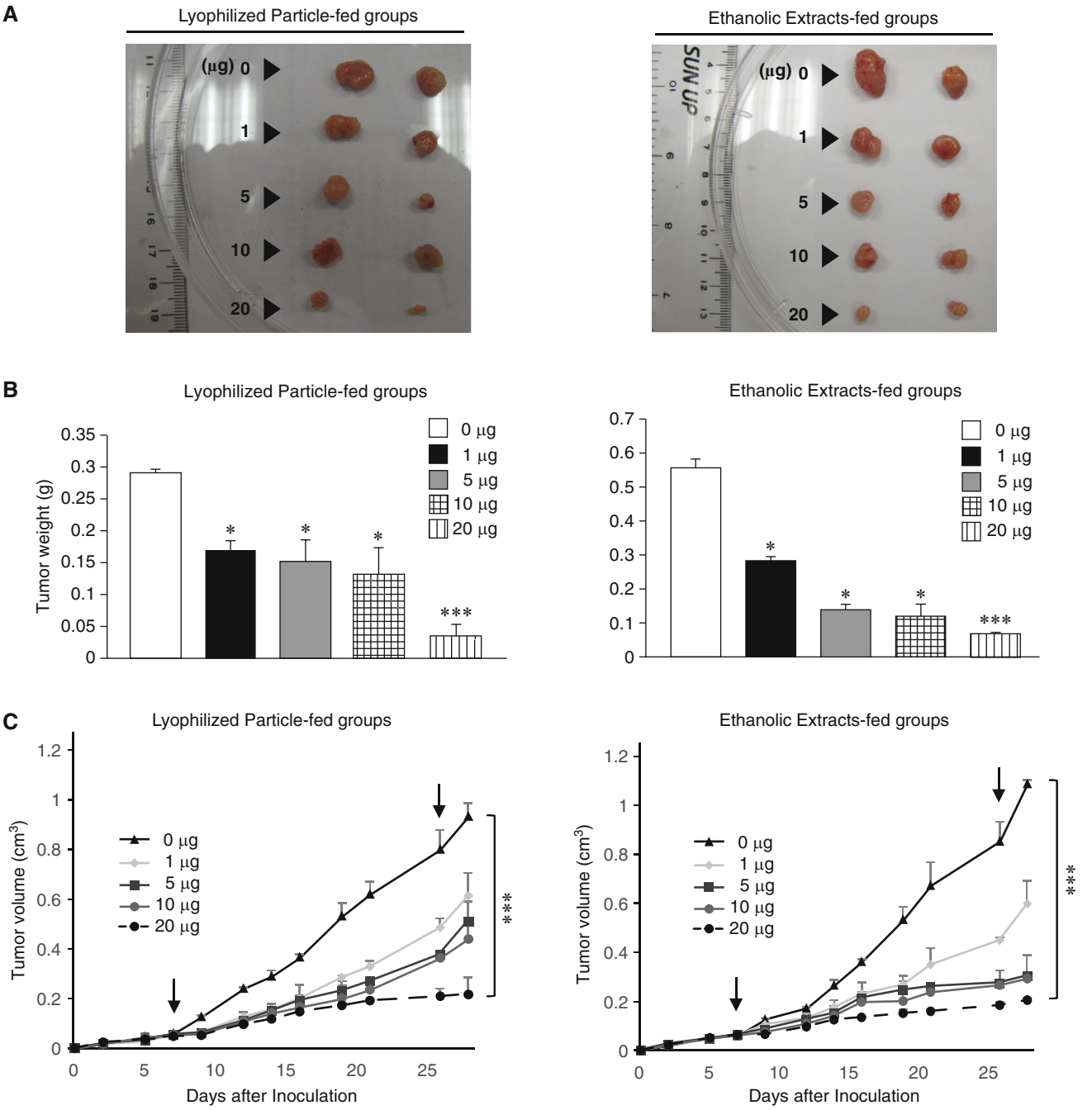

Fig. 2 - ACM or ACME feeding reduced the tumor growth in BALB/c mice injected with SAS cells. Mice were injected with SAS cells followed by feeding with ACM or ACME, and then sacrificed as described in Figure 1. (A) Images of dissected tumors were collected on day 28 from lyophilized particle-fed and ethanolic extracts-fed mice. (the first row: H_2O (control), the second row: treated with 1 μg, the third row: treated with 5 μg, the fourth row: treated with 10 μg and the fifth row: treated with 20 μg). (B) The tumors were removed from lyophilized particle-fed and ethanolic extracts-fed mice and weighed. (C) The tumor growth curves of HNSCC cells in nude mice treated with lyophilized particle and ethanolic extracts were recorded.

an inhibition rate from 22.6% to 65.3%. In the ethanolic extracts-fed group, the inhibition rate was from 18.9% to 54.9%. Intriguingly, neither the lyophilized particles nor the ethanolic extracts treatment caused significant side effects such as a change of gross appearance of organs or body weight in the tumor-bearing mice (Figure 3A and 3B).

4. Discussion

Accumulated evidence has suggested that *Antrodia cinnamomea* could be a potential agent for cancer therapy. For example, Yang *et al* found that the fermented culture broth of *Antrodia cinnamomea* promotes cell cycle arrest and apoptosis of breast

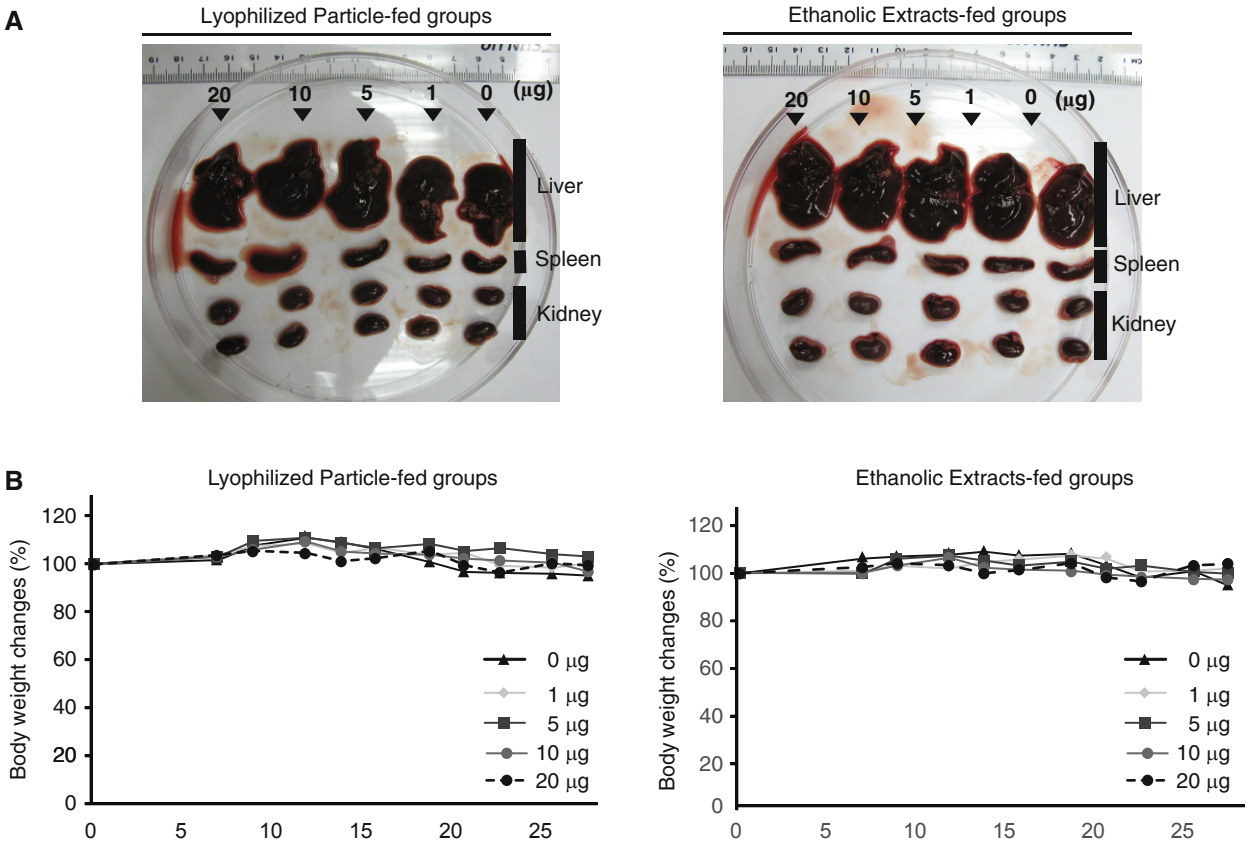

Fig. 3 - Gross appearance of organs and body weight measurement during the ACME feeding model. Gross appearance of organs of mice followed by lyophilized particles or ethanolic extracts treatment after 28 day of tumor development. (B) Measurement of body weight of tumor-bearing nude mice (n = 4) during the course of the lyophilized particles feeding or ethanolic extracts feeding.

cancer cells through suppression of the MAPK signaling pathway [14]. Recently, the anticancer effects of active compounds from *Antrodia cinnamomea* have been identified [6]. Yeh *et al.* demonstrated that a sesquiterpene lactone antrocin from *Antrodia cinnamome* inhibited cell proliferation in non-small-cell lung cancer cells [15]. Yeh *et al.* also demonstrated that a mixture of compounds from *Antrodia cinnamomea* showed a synergistic cytotoxic effect in HT-29 cells [16]. Moreover, in the case of *Antrodia cinnamome*, the anti-cancer efficacy may be attributed to multiple active compounds. But the molecular mechanism and active compounds also need to be studied.

Recent data have demonstrated that cancer cells are functionally heterogeneous and undergo not only proliferation but also differentiation and maturation to a certain degree [17]. Cancer initiating cells (CICs), a more resistant, self-renewing and malignant subpopulation of cancer cells, are considered a novel target in cancer therapy. Elimination of CICs apparently requires exhaustion of stemness and promoting differentiation by targeting self-renewal pathways. Thus, it has been reported that colorectal CICs/CSCs are induced differentiation and their response to chemotherapy can be increased by bone morphogenetic protein 4 (BMP-4) [18]. Moreover, resveratrol, abexinostat and curcumin were previously observed to impair CIC properties, induce CIC differentiation and reduce tumor malignancy through inhibiting self-renewal signaling pathways [19-21]. In our previous study, we demonstrated that HN-CICs possess stemness properties, which are characterized by up-regulation expression of self-renewal gene Oct-4 and Nanog and differentiation ability [22]. Our previous findings found that YMGKI-1, one of the active components from ACME, can diminish tumorigenicity through the blocking of self-renewal ability and induction of CIC differentiation [8]. Together, these studies suggest *Antrodia cinnamome* possesses the ability to target CICs.

In the present study, the anti-tumor activities of lyophilized particles and ethanolic extracts of *Antrodia cinnamomea* mycelia were identified. We found that the tumor-bearing mice which were gavaged with up to 1~20 μg of *Antrodia cinnamomea* mycelium or its extracts three times per week had a reduction of tumor size but did not have organ damage (Figures 2 and 3). Our findings suggest that the product of *Antrodia cinnamomea* mycelia could be a promising adjuvant to conventional treatments for HNSCC that is effective in high risk populations (or patients).

5. Conflict of Interests

No potential conflict of interests was disclosed.

6. Authors' Contributions

Ching-Wen Chang and Yu-Syuan Chen contributed equally to

this article.

7. Acknowledgments

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Case report

Ectodermal dysplasia (ED) syndrome

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ABSTRACT

Ectodermal dysplasia (ED) syndrome comprises a large, heterogeneous group of inherited disorders that are defined by primary defects in the development of 2 or more tissues derived from the embryonic ectoderm. The tissues primarily involved are the skin and its appendages (including hair follicles, eccrine glands, sebaceous glands, nails) and teeth. The clinical features include sparse hair, abnormal or missing teeth, and an inability to sweat due to lack of sweat glands. One such case report of ectodermal dysplasia is presented here.

1. Introduction

Ectodermal dysplasia (ED) syndrome is a rare heterogeneous group of inherited disorders that share primary defects in the development of two or more tissues derived from the ectoderm. These tissues primarily affected are the skin, hair, nails, eccrine glands, and teeth. Defects in tissues derived from other embryologic layers are not uncommon. The disorders are congenital, diffuse, and nonprogressive. To date, more than 192 distinctive syndromes have been described with all possible modes of inheritance. The most common syndromes within this group are hypohidrotic (anhidrotic) ED and hidrotic ED. Hypohidrotic ED (also known as Christ-Siemens-Touraine syndrome) is the more common phenotype and is usually inherited as an X-linked recessive trait. It is characterized by several defects (e.g. hypohidrosis, anomalous dentition, onychodysplasia, hypotrichosis). Typical facies are characterized by frontal bossing, sunken cheeks, a saddle nose, thick and everted lips, wrinkled and hyperpigmented skin around the eyes, and large, low-set ears. Dental manifestations include conical or pegged teeth, hypodontia or complete anodontia, and delayed eruption of permanent teeth. Eccrine sweat glands may be absent or sparse and rudimentary, particularly in those with hypohidrotic ED. In some cases, mucous glands are absent in the upper respiratory tract and in the bronchi, esophagus, and duodenum. Scalp hair may be sparse, short, fine, dry, or there may be a complete absence of hair. Structural hair-shaft abnormalities like longitudinal grooving, hair-shaft torsion, and cuticle ruffling can also be seen. The prevalence of atopic eczema is high. Other common signs are short stature, eye abnormalities, decreased tearing, and photophobia. Intelligence in those affected is normal. Nails are often brittle and thin or show abnormal ridging, but they may be grossly deformed especially in the hidrotic type. The presence or absence of these abnormalities defines the different types of this syndrome.

2. Case report

A 27-year-old man was presented to the outpatient department with hypohidrosis and scant body hair. He had a past medical history of asthma with frequent attacks and regularly inhaled corticosteroid and bronchodilator. Easy hyperthermia with hypohidrosis was noted during childhood. He also had malformed teeth all with a cone-shaped aspect (Figure 1), but he received teeth replacements 10 years ago. The clinical phenotype of the patient was characterized by frontal bossing, a prominent supraorbital ridge, sunken cheeks, thick lips, sparse hair, scanty eyebrows, and low-set and overfolding ears (Figure 2). A physical examination showed hypotrichosis with fine, sparse and brittle scalp hair with excessive fragility (Figure 3). Body hair was also diminished and sweat glands were found only over the axilla region. Sexual hair, beard and pubic hair, was normal. The patient also had generalized dry and hypopigmented skin with reduced hair follicles. Dermatitis resembling atopic skin was mainly noted over his bilateral extremities. None of his family member had similar symptoms. Hypohidrotic ED was impressed.

3. Discussion

ED syndrome is a group of genetic disorders identified by lack or dysgenesis of at least two ectodermal derivatives such as hair, nails, teeth, or sweat glands. Obvious manifestations of the disorders are not clinically apparent in newborns. They normally

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Fig. 1 - The patient was 5 year-old with obvious cone-shape teeth and scanty eyebrows.

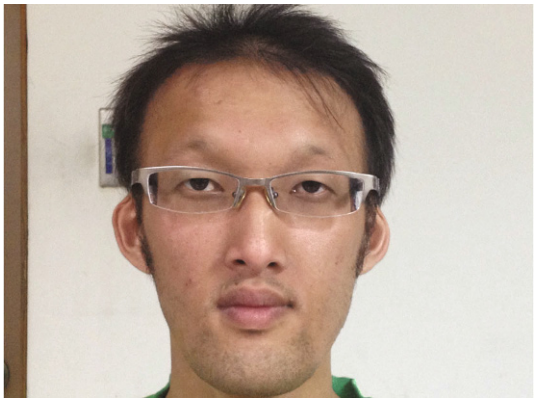

Fig. 2 - Frontal bossing, prominent supraorbital ridge, sunken cheeks, thick lips, low-set ears, scanty eyebrows and hypotrichosis with fine, sparse and brittle scalp hair but normal sexual hair (beard).

become evident during infancy or early childhood. The patient reported here had involvement of hair, sweat glands, and teeth. Other ectodermal structures were largely unaffected. In addition, he had atopic eczema, frontal bossing, sunken cheeks and low set ears. These clinical features were supportive in diagnosing hypohidrotic ED. Currently, 64 genes and 3 chromosomal loci [1] have been identified in about 62 different ED syndromes. Hypohidrotic ED is the most frequent form of ED syndrome that can be inherited in an X-linked (XL), autosomal recessive (AR) or (AD) autosomal dominant manner. The XL recessive type is the most common form of hypohidrotic ED. 94% of the patients carries the mutation of the *EDA1* gene. This defective gene was cloned thereby leading to the identification of a novel signaling molecule of the tumor necrosis factor (TNF) superfamily named ectodysplasin (EDA) [2]. Autosomal forms of hypohidrotic ED are due to mutations in the EDA receptor (EDAR), a novel TNF receptor family member. Currently over 100 different mutations in the EDA gene have been reported, while only 20 causative mutations are known for EDAR. Morbidity and mortality are related to the absence or presence of eccrine and mucous glands. Children with decreased

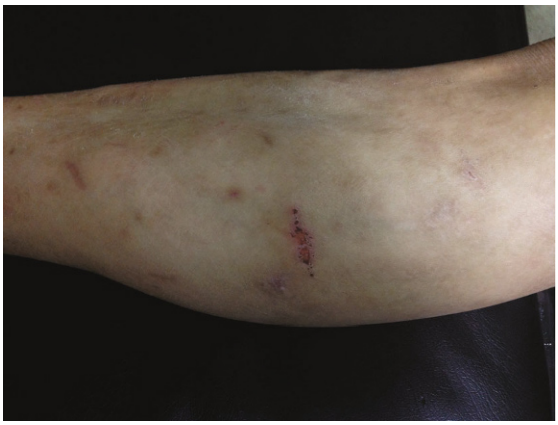

Fig. 3 - Fragile-appearing dry skin with reduced hair follicles.

sweating may have a mortality rate of up to 30% in infancy or early childhood because of intermittent hyperpyrexia. No definite pharmacological treatment is available, and the management of affected patients depends on which structures are involved. Patients with hypohidrotic ED are advised to wear light clothing, to carry a cold-water spray bottle, and to stay in air conditioned environments whenever possible. For patients with dental defects, early dental evaluation and intervention is advised, as is routine dental hygiene. Orthodontic treatment may be undertaken for cosmetic reasons and to ensure adequate nutritional intake. Life expectancy in such cases where the necessary precautions are observed, is almost as good as in other, non-affected children.

4. Conclusion

Ectodermal dysplasia syndrome is a rare genetic disorder with the involvement of various tissues in the body. A careful and a thorough examination of a patient will lead to an accurate diagnosis. It should be noted that an absence of a positive family history for ectodermal dysplasia should not be a factor in causing any diagnostic dilemmas with respect to ectodermal dysplasia, a condition that shows multiple modes of inheritance.

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