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Natural Flavonoids in StAR Gene Expression and Testosterone Biosynthesis in Leydig Cell Aging

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1. Introduction

It is well known that blood testosterone level declines during the course of male aging (Feldman et al., 2002; Harman et al., 2001), a phenomena that is associated with the decreases in bone density, muscle mass and strength, sexual function and other physiological parameters (Kaufman & Vermeulen, 2005; Matsumoto, 2002; Vermeulen, 2000). Previous studies reported that serum testosterone concentrations were lower in the male patients with Alzheimer's disease in comparison to non-demented and age-matched men (Hogervorst et al., 2001; Moffat et al., 2004). Further studies observed that supplementation with testosterone in rats reduced β-amyloid peptide and hyperphosphorylation of τ-protein, two biomarkers of the disease (Gouras et al., 2000; Papasozomenos & Shanavas, 2002; Ramsden et al., 2003). The studies suggest that low blood testosterone is a possible risk factor for the development of Alzheimer's disease (Rosario & Pike, 2008). The decline in blood testosterone is a progressive process in male aging. Several longitudinal studies on the blood testosterone of aging males indicated that the incidence of hypogonadism increased with age (Feldman et al., 2002; Harman et al., 2001). In addition, many pathological and stress-related factors may accelerate this process. Therefore, delaying the decline in blood testosterone is clinically significant for the health of aging males suffering from hypogonadism.

For delaying the decline in testosterone, understanding the mechanisms responsible for the decline is important. The studies in the last decades reported multiple factors and alterations in aging process that affect the levels of blood testosterone (Wang & Stocco, 2005). The studies further indicated that the primary reason for the decline is the decrease in testosterone biosynthesis during male aging (Chen et al., 1994). Testosterone is principally synthesized in testicular Leydig cells from the substrate cholesterol and released into the blood circulation (Miller, 1988). The rate-limiting step in testosterone biosynthesis is the transfer of cholesterol to the mitochondrial inner membrane to initiate the steroidogenic process in Leydig cells (Stocco & Clark, 1996). This step is regulated by the steroidogenic acute regulatory (StAR) protein, a critical factor in steroid hormone biosynthesis that controls the cholesterol transfer to the mitochondrial inner membrane (Clark et al., 1994; Lin et al., 1995; Wang et al., 1998). However, StAR protein also declines in Leydig cell aging and

the mitochondrial cholesterol transfer is defective in aged Leydig cells (Culty et al., 2002; Leers-Sucheta et al., 1999; Luo et al., 2001). Therefore, understanding the mechanism for the decline in StAR gene expression becomes an important consideration in the research on the age-related decline in testosterone biosynthesis.

Further studies observed that cyclooxygenase-2 (COX2) in Leydig cells increased in male aging, and the increase in COX2 depressed StAR gene expression and testosterone production. Inhibition of COX2 activity in aged Leydig cells reversed the declines in StAR protein and testosterone production. In addition, feeding aged rats with a COX2 inhibitor reversed the decreased StAR protein and blood testosterone concentrations (Wang et al., 2005). These observations suggest a novel mechanism involving COX2 in the age-related decline in testosterone biosynthesis. The results from the studies indicate that it is possible to delay the decline using COX2 inhibitors. However, aging is a long process and long-term application of pharmacological levels of COX2 inhibitors is limited by their potential side effects. In search for safe and practical approaches, a group of natural flavonoids in food and food supplements has been identified being able to block the COX2-dependent signaling. These flavonoids acted at the different steps of the COX2 signaling pathway and significantly enhanced StAR gene expression and testosterone biosynthesis in Leydig cells. This chapter will describe the mechanism involving COX2 in the declines in StAR gene expression and testosterone biosynthesis in Leydig cell aging. The effects of the flavonoids on this mechanism will be reviewed. The potential application of the natural flavonoids in delaying the declines in StAR gene expression and testosterone biosynthesis will be discussed.

2. Luteinizing hormone-induced signaling in StAR gene expression and testosterone biosynthesis in Leydig cell aging

StAR gene expression and testosterone biosynthesis in testicular Leydig cells are mainly regulated by luteinizing hormone (LH) secreted from pituitary gland. LH stimulation of Leydig cells induces two messengers: cyclic AMP (cAMP) and arachidonic acid (AA). These two messengers transduce signals to the nucleus through two separated pathways to co-regulate StAR gene expression. Both pathways are required with neither one alone being sufficient for LH-induced StAR gene expression and testosterone production (Wang & Stocco, 1999). In addition, these two pathways transduce both positive and negative signals to regulate StAR gene expression. The negative signals increase in Leydig cell aging, resulting in decreases in StAR gene expression and testosterone biosynthesis.

2.1 LH-induced positive signaling through cAMP-protein kinase A pathway

It is documented that LH-induced signaling through cAMP-protein kinase A (PKA) pathway phosphorylates the transcription factors regulating StAR gene expression (Reinhart et al., 1999b). PKA-phosphorylation is important for the activities of several groups of transcription factors, including cAMP-responsive element binding (CREB) protein, steroidogenic factor-1 (SF-1) and GATA-4, which bind on StAR promoter to induce StAR gene transcription. The cAMP-responsive element (CRE)-like sites were found in StAR promoter DNA sequences. The results from electrophoretic mobility shift assays (EMSA) demonstrated the binding of CREB to StAR promoter (Manna et al., 2002; Manna et al., 2003). PKA-phosphorylation is critical for the activity of CREB (Meyer & Habener, 1993; Montminy et al., 1986), because the phosphorylation of CREB is needed for the recruitment

of its cofactor CBP (CREB binding protein) that is involved in the transcriptional activity of CREB (Chrivia et al., 1993; Mayr & Montminy, 2001; Mayr et al., 2001). SF-1 binding sites were also located on StAR promoter (Sandhoff et al., 1998). Binding of SF-1 to these binding sites is crucial for StAR gene transcription (Caron et al., 1997a; Caron et al., 1997b; Sandhoff et al., 1998; Sugawara et al., 1997). PKA-phosphorylation of SF-1 protein was detected at serine and threonine residues (Zhang & Mellon, 1996), which was further confirmed by the study with R2C rat Leydig cells (Carlone & Richards, 1997). It appears that PKA-phosphorylation is needed for the maximal activity of SF-1 in the regulation of StAR gene transcription (Lopez et al., 2001; Sugawara et al., 1996). GATA-4 is another transcription factor binding on StAR promoter to regulate StAR gene expression. A GATA-4 binding site was found at -61 to -66 in StAR promoter sequences (Silverman et al., 1999; Silverman et al., 2006). Stimulation of MA-10 mouse Leydig cells with cAMP dramatically increased phosphorylated GATA-4 protein (Tremblay et al., 2002), resulting in the increase in StAR promoter activity. The essential role of PKA-phosphorylation in the activity of GATA-4 was further confirmed by over-expression of PKA catalytic subunit. While expression of PKA catalytic subunit significantly increased GATA-4-supported StAR promoter activity, the increased promoter activity was reversed by co-expression of the protein kinase inhibitor (Tremblay et al., 2002).

The signaling through cAMP-PKA pathway is able to increase the activities of a group of transcription factors that bind on StAR promoter and regulate StAR gene transcription, including C/EBPβ (Christenson et al., 1999), Sp-1 (Sugawara et al., 2000), activator protein-1 (AP-1) (Manna et al., 2004), and sterol regulatory element binding protein (SREBP) (Shea-Eaton et al., 2001), by inducing the interaction and cooperation among them. The cAMP or PKA-induced interactions among these transcription factors generated synergistic effects that increased the cAMP-induced StAR gene transcription and steroid hormone production in Leydig cells (Reinhart et al., 1999a; Silverman et al., 1999; Silverman et al., 2006; Sugawara et al., 2000; Tremblay et al., 2002).

In addition, it was observed that cAMP stimulation of Leydig cells reduced DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) protein. DAX-1 is a strong transcriptional repressor that binds to a hairpin structure of StAR promoter between -20 to -61 and depresses StAR gene expression (Zazopoulos et al., 1997). The reduction of DAX-1 by cAMP stimulation results in dramatic increases in StAR gene expression and steroid hormone production in Leydig cells (Jo & Stocco, 2004). Although it is still not clear how the signaling through cAMP-PKA pathway reduces DAX-1 protein, the observations indicate that this signaling pathway generates positive signals to increase StAR gene transcription by reduction of the transcriptional repressor.

2.2 LH-induced positive signaling through AA pathway

In addition to the cAMP-PKA signaling pathway, LH induces positive signaling through AA pathway. Stimulation of Leydig cells with LH induced AA release in one minute (Cooke et al., 1991). Previous studies have described three mechanisms for the AA release by LH or hCG: 1) Binding of the trophic hormones to their receptors activates G protein. G protein directly activates phospholipase A2 (PLA2) that catalyzes AA release from phospholipids (Axelrod et al., 1988; Ronco et al., 2002); 2) G protein increases cAMP formation that induces AA release, possibly through the activation of PLA2 by PKA-phosphorylation (Piomelli & Greengard, 1991; Wang et al., 2002); 3) AA is released by the co-regulation of acyl-CoA synthetase 4 (ACS-4) and acyl-CoA thioesterase (Acot2). Trophic hormone stimulation

induces ACS-4, and activates Acot2 by cAMP-PKA-phosphorylation. Co-action of these two enzymes catalyzes AA release from arachidonyl CoA (Cano et al., 2006; Cornejo Maciel et al., 2005; Finkielstein et al., 1998; Maloberti et al., 2005; Maloberti et al., 2007; Paz et al., 1994). The LH-induced AA release is required for testosterone biosynthesis. It was observed that incubation of Leydig cells with AA significantly increased testosterone production (Didolkar & Sundaram, 1987, 1989). When the AA release was blocked using PLA2 inhibitors, the LH-induced testosterone production was significantly reduced although the inhibitors did not affect the cAMP formation (Abayasekara et al., 1990). Inhibiting AA metabolism in rat Leydig cells produce similar results (Cooke et al., 1984; Dix et al., 1984; Mele et al., 1997; Sullivan & Cooke, 1985). In addition, blocking AA release by knockdown of the gene expressions of ACS-4 and Acot2 significantly reduced steroidogenesis (Maloberti et al., 2005). The studies indicated that LH induces positive signaling through AA pathway to regulate testosterone biosynthesis in Leydig cells.

To understand how the released AA plays such an important role in LH-induced steroidogenesis in Leydig cells, its effect on mitochondrial cholesterol transfer was investigated. It was found that blocking AA release failed to affect steroidogenesis when Leydig cells were incubated with 22(R)hydroxycholesterol (Abayasekara et al., 1990; Dix et al., 1984; Mele et al., 1997), a water soluble substrate that is able to diffuse to the mitochondrial inner membrane. The studies suggest that AA enhance testosterone biosynthesis by increasing the mitochondrial cholesterol transfer. The observations were corroborated by the increases in StAR protein and steroidogenesis in the Leydig cells incubated with AA. In addition, blocking AA release using PLA2 inhibitors dramatically reduced LH- or cAMP-induced StAR promoter activity, StAR mRNA, StAR protein and steroidogenesis in Leydig cells. Importantly, the reduced StAR gene expression and steroidogenesis by PLA2 inhibitor were reversed by addition of AA in the cell cultures (Wang et al., 2000; Wang et al., 1999). It is clear that AA increases testosterone biosynthesis by enhancing StAR gene expression. Moreover, the results from EMSA show that AA enhanced the binding of nuclear protein(s) to the StAR promoter DNA sequences between -67 and -96 (Wang et al., 2003a). The observation indicates that AA acts on StAR promoter and regulates StAR gene transcription.

AA is metabolized mainly through three metabolic pathways, the lipoxygenase, epoxygenase and cyclooxygenase pathways, generating various metabolites. To determine which pathways are involved in the LH-induced StAR gene expression and steroidogenesis, MA-10 Leydig cells pre-loaded with ^{3}H-AA were used to study the effects of cAMP on AA metabolism. The results from HPLC analysis of the cell extract showed that cAMP stimulation significantly increased two groups of AA metabolites: the lipoxygenase-generated metabolites, 5-hydroxyeicosatetraenoic acid (5-HETE) and 5-hydroperoxyeicosatetraenoic acid (5-HPETE) (Wang et al., 2003a); and the epoxygenase-generated metabolites, 5,6-epoxyeicosatrienoic acid (5,6-EET), 8,9-epoxyeicosatrienoic acid (8,9-EET), and 11,12-epoxyeicosatrienoic acid (11,12-EET) (Wang et al., 2006). The roles of these AA metabolites in StAR gene expression were investigated with Leydig cells. It was observed that addition of each of these metabolites into the cultures of Leydig cells significantly increased cAMP-induced StAR promoter activity, StAR mRNA, StAR protein and steroidogenesis. In addition, when activity of lipoxygenase or epoxygenase was inhibited, the cAMP-induced StAR gene expression and steroidogenesis were significantly reduced (Wang et al., 2000). The observations indicated that each of the metabolites enhanced StAR gene expression at the level of transcription. Although how these AA

metabolites act on StAR promoter is not clear, the studies showed that LH- or cAMP-released AA is converted to positive signals, 5-HPETE, 5-HETE, 5,6-EET, 8,9-EET and 11,12-EET through lipoxygenase and epoxygenase pathways, which enhance StAR gene expression and testosterone biosynthesis in Leydig cells.

2.3 LH-induced negative signaling in StAR gene expression

Whereas LH-released AA is metabolized to the positive signals by lipoxygenase and epoxygenase, it is converted to negative signals by cyclooxygenase (COX). It was found that inhibition of COX activity using an inhibitor significantly increased cAMP-induced StAR protein and steroid hormone production in MA-10 mouse Leydig cells. Similar increases were observed in StAR promoter activity and StAR mRNA levels. Although inhibition of COX activity significantly increased the cAMP-induced StAR gene expression, in the absence of cAMP the inhibitor alone did not increase StAR protein expression and steroid hormone production. The studies suggest that the COX inhibitor itself is not able to induce StAR gene expression, but rather reduce the negative signals, resulting in significant increases in the cAMP-induced StAR gene expression and steroidogenesis. There are two isoforms of COX enzymes, COX1 and COX2, in Leydig cells. To determine which isoform is responsible for the inhibitory effect on StAR gene expression, Leydig cells were treated with the selective COX1 inhibitor SC560 or COX2 inhibitor NS398, respectively. Whereas inhibition of COX1 activity did not change StAR protein level, inhibition of COX2 activity dramatically enhanced cAMP-induced StAR protein expression and steroid hormone production. Further studies observed that while COX2 inhibitor significantly increased StAR protein, it did not affect PKA activity. In addition, over-expression of COX2 reduced StAR promoter activity. The observations indicate that COX2, not COX1, converts the LH- or cAMP-released AA to negative signals that depress StAR gene transcription in Leydig cells (Wang et al., 2003b).

The signaling through cAMP-PKA pathway also generates negative signals to inhibit StAR gene expression and steroidogenesis in Leydig cells by increasing COX2 expression. The mechanism responsible for the cAMP-increased COX2 may involve the signaling through cAMP-PKA pathway in the activation of several transcription factors that regulate COX2 promoter activity. One is CREB that binds on the CRE site at -53 to -59 of human COX2 promoter sequences (Schroer et al., 2002). CREB activation by PKA increased its binding to the CRE site and induced COX2 gene transcription. While forskolin or cAMP activated PKA and increased COX2 promoter activity, inhibition of PKA activity reversed the increase in the promoter activity. Another transcription factor NF-κB plays an essential role in regulating COX2 gene expression (Arun et al., 2009). This transcription factor was activated by PKA-phosphorylation of NF-κB p65 sub-unit. Inhibition of its phosphorylation using H89 reduced COX2 gene transcription. The involvement of cAMP-PKA signaling pathway in the LH-induced COX2 expression was supported by the studies with Leydig cells. An earlier study reported that hCG stimulation of Leydig cells increased COX2 expression (Frungieri et al., 2006). This observation was corroborated by a latter study that reported a LH-increased COX2 protein in rat Leydig cells. The increase in COX2 was almost abolished by inhibition of PKA activity with its inhibitor H89, indicating that cAMP-PKA-phosphorylation is required for the LH-induced COX2 expression in Leydig cells (Chen et al., 2007b).

As LH stimulates testosterone biosynthesis, the increase in testosterone may feed back to induce COX2 expression in Leydig cells. A recent study reported that testosterone induced COX2 expression and prostaglandin F2a production in hamster Leydig cells (Matzkin et al.,

2009). These effects of testosterone were abolished by the anti-androgen bicalutamide. A testosterone-stimulated phosphorylation of the mitogen-activated protein kinase (MAPK) was also observed in the study. It is known that the activity of MAPK is important in COX2 gene expression (Chun & Surh, 2004), because MAPK is able to activate the transcription factors that regulate COX2 gene transcription.

The studies described above indicate that when LH stimulates positive signals through cAMP-PKA pathway and AA pathway, it also induces negative signals through these two pathways. Therefore, the total effect of LH on StAR gene expression and testosterone biosynthesis depends on the ratio and levels of the positive and negative signals. The alterations of the signals in Leydig cell aging affect StAR gene expression and testosterone biosynthesis.

2.4 The age-related alterations of the positive and negative signals in StAR gene expression

Previous studies have reported the multiple alterations or defects during male aging that are involved in the decline in testosterone biosynthesis (Wang & Stocco, 2005). Many of these alterations or defects in the aging process increase the negative signals and reduce the positive signals in StAR gene expression. These changes reduce the sensitivity of Leydig cells to trophic hormone stimulation and result in the declines in StAR gene expression and testosterone biosynthesis.

It was observed that the age-related alteration in hypothalamic-pituitary-adrenal axis attenuates the sensitivity of the feedback control of adrenal steroidogenesis, resulting in the increases in basal and stress-induced glucocorticoid levels (Hardy & Cooper, 2010). The inhibitory effect of glucocorticoid on testosterone biosynthesis was reported by previous studies with rat Leydig cells. This steroid hormone was shown being able to inhibit PLA2 activity and block LH-induced AA release (Abayasekara et al., 1990). Further studies observed that blocking AA release using dexamethasone dramatically reduced StAR protein and steroidogenesis in MA-10 mouse Leydig cells. Similar results were obtained in the analyses of StAR promoter activity and StAR mRNA levels. The reduced StAR gene expression and steroidogenesis were reversed by addition of AA to the cell cultures (Wang et al., 2000). The studies suggest that the age-related defect in hypothalamic-pituitary-adrenal axis result in reduction in the LH-induced positive signals through AA pathway.

The positive signaling through cAMP-PKA pathway is also reduced in Leydig cell aging. The previous studies with aged Leydig cells reported a defect in this signaling pathway that caused the decreases in LH-induced cAMP formation and PKA activity (Lin et al., 1980; Luo et al., 2005). The decrease in LH-induced cAMP formation may significantly contribute to the reduced sensitivity of aged Leydig cells to LH stimulation. The mechanism for this defect in aged Leydig cells has not been completely understood. It was observed that the number of the LH-binding sites and their binding affinity decreased in aged Leydig cells, but how the reduced LH-binding capacity affected the cAMP formation remains to be elucidated (Chen et al., 2002). In addition, defect of G protein was investigated. It is possible that inefficiency of G protein or defect in coupling among the signal cascades affects the LH-induced cAMP formation (Chen et al., 2004).

In the aging process, the levels of many biological factors in blood or testis are altered. These alterations may reduce the positive signaling through cAMP-PKA pathway and enhance the negative signaling through COX2 pathway. One group of the biological factors that increase in aging is the inflammatory cytokines, such as Interlukin-1 (IL-1), Transforming growth factor

(TGF)-β and tumor necrosis factor (TNF) (Chung et al., 2001; Morley & Baumgartner, 2004). IL-1β was reported to be inhibitory in hCG-, cAMP-, and forskolin-induced testosterone production (Calkins et al., 1990). While it is able to reduce activities of steroidogenic enzymes (Hales, 1992; Lin et al., 1991), IL-1β induced COX2 expression (Chen et al., 2007b; Walch & Morris, 2002) and reduced StAR protein expression (Ogilvie et al., 1999). TGF-β in testis increases with age (Jung et al., 2004). The increase in TGF-β may significantly reduce trophic hormone-induced cAMP formation and testosterone production in Leydig cells (Avallet et al., 1987). TNF-α reduced hCG-stimulated cAMP formation in a concentration-dependent manner in MA-10 mouse Leydig cells (Budnik et al., 1999), which was associated with a reduction in StAR protein. In addition, it was reported that the transcription factor NF-kB increases in the aging process (Chung et al., 2009; Chung et al., 2006). This increase would contribute to the increase in COX2, because NF-kB plays an essential role in COX2 gene transcription. Another group of the biological factors is reactive oxidative species (ROS). An age-related increase in ROS was previously described (Chen et al., 2001b). While ROS may inhibit testosterone through different mechanisms, it is able to activate NF-kB, resulting in the increases in COX2 and various inflammatory factors (Chung et al., 2006).

The age-related increases in the biological factors, such as IL-1β, NF-kB and ROS, suggest an increase in COX2 expression in Leydig cell aging, because these factors induce COX2 as mentioned above. This was supported by the increase in COX2 mRNA level detected in aged Leydig cells of rats (Syntin et al., 2001). It is known that COX2 produces an inhibitory effect on StAR gene expression and steroidogenesis (Wang et al., 2003b). These observations suggest a possibility of involving an age-related increase of COX2 in the declines in StAR gene expression and testosterone production. This possibility was demonstrated by the studies with aged rats (Wang et al., 2005). It was found that from 3 to 30 months of age, the levels of COX2 protein in aged rat Leydig cells increased by 346% over that of young Leydig cells. The increase in COX2 was associated with the decreases in StAR protein, testosterone biosynthesis, and blood testosterone concentration in aged rats. Inhibition of COX2 activity with the selective COX2 inhibitor NS398 reversed the decreases in StAR protein and testosterone production in aged Leydig cells. In addition, while over-expression of COX2 reduced cAMP-stimulated StAR protein and steroidogenesis, co-incubation with NS398 reversed the reduced StAR protein and steroid hormone. These findings reveal a novel mechanism in the age-related declines in testosterone biosynthesis.

3. Mechanism for COX2-dependent inhibition of StAR gene expression and testosterone biosynthesis in Leydig cell aging

The studies described above indicated an involvement of COX2 in the age-related declines in StAR gene expression and testosterone biosynthesis. However, the mechanism for the COX2-dependent inhibition of StAR gene expression in Leydig cells needs to be elucidated. While further investigations are needed for the elucidation, the studies in the recent years have significantly improved the understanding of the mechanism.

3.1 AA distribution between COX2 and lipoxygenase metabolic pathways

It is known that the LH-released AA is converted to positive signals through lipoxygenase and epoxygenase pathways (Wang et al., 2006; Wang et al., 2003a), and also negative signals through COX2 pathway (Wang et al., 2003b). This suggests that StAR gene expression and testosterone biosynthesis in Leydig cell aging are regulated by the activities of these AA

metabolic enzymes, because they control the ratio of the positive and negative signals. To prove this hypothesis, MA-10 mouse Leydig cells pre-loaded with ^{3}H-AA were incubated with NS398 to inhibit their COX2 activities. AA metabolites were extracted from the cells and analyzed by HPLC. The HPLC profile of AA metabolites indicated that inhibition of COX2 activity increased AA metabolites produced through lipoxygenase pathway, with the levels of 5-HETE and 5-HPETE being significantly increased by the treatment. Incubation of rat Leydig cells with 5-HETE significantly increased testosterone production (Wang et al., 2005). It was previously reported that 5-HETE and 5-HPETE transduce positive signals to enhance StAR gene expression and steroidogenesis in Leydig cells (Wang et al., 2003a). The observations indicated that the distribution of AA between these two metabolic pathways is regulated by their enzyme activities. Thus, inhibition of COX2 activity changed the AA distribution, resulting in the increases in 5-HETE and 5-HPETE that enhanced StAR gene expression. It is possible that as COX2 expression increases in aged Leydig cells, more AA is metabolized through COX2 pathway to produce inhibitory metabolites that depress StAR gene expression and testosterone biosynthesis.

3.2 The signaling through COX2-prostaglandin F2α-receptor pathway in StAR gene expression

In the COX2 metabolic pathway, COX2 catalyzes AA to prostsglandin(PG)H2 that is further metabolized to PGD2, PGE2, PGF2α, PGI2 and thromboxane A2 (TBX A2) by different prostaglandin synthases and thromboxane A synthase (TBXAS). Among these metabolites, PGF2α is involved in the COX2-dependent inhibition of testosterone biosynthesis. Previous studies reported the hCG-induced production of PGF2α (Haour et al., 1979), which acted as a negative signal to inhibit testosterone production in Leydig cells (Bartke et al., 1973; Saksena et al., 1973). The inhibitory effect of PGF2α was verified by several studies. In an *in vivo* study, the infusion of PGF2α to rats (250μg/rat) for 4 hours reduced blood testosterone to 50%, whereas the inhibition of PGF2α production with indomethacin increased blood testosterone concentrations. When the decapsulated testis preparation was incubated with PGF2α, LH-induced testosterone production was reduced in a dose-dependent manner (Fuchs & Chantharaksri, 1981). The observations were corroborated by the additional studies on the inhibitory effects of PGF2α on Leydig cell steroidogenesis (Romanelli et al., 1995; Sawada et al., 1994). To find how PGF2α inhibits testosterone biosynthesis, the PGF2α receptors were located on hamster Leydig cells. Further investigation observed that incubation of the Leydig cells with 1 μM of PGF2α for 10 to 60 minutes significantly reduced StAR protein. The study described a COX-2-depenednt signaling pathway via COX2, PGF2α production, PGF2α receptors, and StAR protein in the regulation of hCG-induced testosterone biosynthesis (Frungieri et al., 2006). How this signaling pathway regulates StAR protein expression is not clear. To understand the mechanism, several regions in rat StAR promoter DNA sequences were detected to be responsive to PGF2α, including the bind sites of DAX-1, c-Fos and YY1. DAX-1 is described above as a transcriptional repressor. Injection of PGF2α for 2 hours resulted in the increase in ovarian DAX-1 protein. The increase in DAX-1 protein was associated with the 50% reduction in StAR mRNA (Sandhoff & McLean, 1999). The c-Fos binding sites were located at -85, -187 and -1561 of StAR promoter sequences. Administration of PGF2α increased c-Fos expression and reduced StAR gene transcription (Shea-Eaton et al., 2002). The YY1 binding sites were found in StAR promoter sequences at -1230/-1238, -1550/-1559 and -1651/-1660. PGF2α enhanced ovarian YY1 expression, resulting in a decrease in StAR gene expression (Liu et al., 2007; Nackley et al.,

2002). However, whether PGF2α increases the expressions of these transcriptional repressors in Leydig cells remains to be clarified.

3.3 The signaling through COX2-TBXAS-TBX A2-receptor pathway in StAR gene expression

Following the studies on PGF2α, TBXAS activity was inhibited to examine the role of the COX2-TBXAS-TBX A2-receptor signaling pathway in Leydig cell steroidogenesis. Inhibition of TBXAS activity with the inhibitor furegrelate significantly enhanced the cAMP-induced steroidogenesis in MA-10 mouse Leydig cells. However, co-incubation of the cells with 22(R)hydroxycholesterol abolished the stimulatory effect of the inhibitor on steroidogenesis, suggesting that TBXAS produce an inhibitory effect on the mitochondrial cholesterol transfer. The results were confirmed by the increase in StAR protein in the cells treated with the TBXAS inhibitor (Wang et al., 2008). Following these observations, luciferase assays of StAR promoter activity and reverse transcription-polymerase chain reaction (RT-PCR) were performed to determine whether the TBXAS inhibitor acted on StAR gene transcription. It was found that inhibition of TBXAS activity significantly increased StAR promoter activity and StAR mRNA levels. To confirm the inhibitory effect of TBXAS on StAR gene expression, the TBXAS gene was silenced using RNA interference (RNAi). As TBXAS gene was silenced by RNAi, StAR mRNA, StAR protein and steroid hormone production in the cells were significantly increased. It is clear that the signaling through COX2 and TBXAS acts on StAR promoter and depresses StAR gene transcription. The study was continued to identify the transcription factor(s) that is affected by the activity of TBXAS. The results from the study reveal that inhibition of TBXAS activity reduced DAX-1 protein. When DAX-1 protein was reduced by inhibiting TBXAS activity, StAR gene transcription was significantly enhanced, suggesting that co-action of COX2 and TBXAS convert AA to inhibitory metabolite(s) that inhibits StAR gene transcription by regulating DAX-1 expression (Wang et al., 2008).

The first AA metabolite in the COX2-TBXAS pathway is TBX A2. The TBX A2 receptors were detected in several mouse Leydig cell lines and identified on the cell surface (Pandey et al., 2009). The specific binding of the receptor antagonist SQ29548 to the receptors on MA-10 mouse Leydig cells was demonstrated by binding assay and the binding competition between ^{3}H-SQ29548 and another receptor antagonist BM567. When the concentrations of BM567 increased from 0 to 10 μM, the ^{3}H-SQ29548 bound to the cells was reduced to 3%. The receptor antagonist SQ29548 was used to block the binding of TBX A2 to the receptors and to determine its effect on the Leydig cell steroidogenesis. It was observed that blocking the TBX A2 receptors on the Leydig cells incubated with 0.1 mM cAMP dramatically increased StAR protein in a concentration-dependent manner. The increase in StAR protein was paralleled with the increase in steroid hormone production, with progesterone production being increased from 35 to 208 pg/μg cellular protein as the concentrations of SQ29548 were increased from 0 to 25 μM. The results were verified using another receptor antagonist BM567 (Pandey et al., 2009). Since the COX2-dependent signaling is involved in the age-related declines in StAR gene expression and testosterone biosynthesis (Wang et al., 2005), aged Leydig cells were used to determine whether blocking the TBX A2 receptors is able to reversed the declines. When the TBX A2 receptors were blocked with SQ29548, StAR protein in the aged Leydig cells increased significantly. In concomitant with the increase in StAR protein, testosterone production by the aged Leydig cells also significantly increased from 86 to 146 pg/μg cellular protein (Pandey et al., 2009).

To understand how the receptor antagonists enhanced StAR protein expression and steroidogenesis, their effects on StAR gene transcription were investigated. Luciferase assays of StAR promoter activity indicated that blocking the TBX A2 receptors with the antagonists enhanced StAR promoter activity in a concentration-dependent manner in the MA-10 cells incubated with 0.1 mM cAMP. The increase in the promoter activity was associated with a similar increase in StAR mRNA level (Pandey et al., 2009). The observations indicated that blocking the TBX A2 receptor enhanced StAR gene transcription, similar to the observations on the cells treated with TBXAS inhibitor (Wang et al., 2008). Further study showed that blocking the TBX A2 receptors significantly reduced DAX-1 protein and increased StAR protein in MA-10 mouse Leydig cells. The results were confirmed with the Leydig cells isolated from aged rats. The aged Leydig cells expressed high levels of DAX-1 protein, but blocking the TBX A2 receptors dramatically reduced the DAX-1 protein, which was associated with the increases in StAR protein and testosterone production (Pandey et al., 2009). These observations indicated that the signaling through COX2-TBXAS-TBX A2-receptor inhibits StAR gene expression and testosterone biosynthesis by regulation of DAX-1 expression.

In addition, while sub-threshold levels of cAMP were unable to stimulate significant increases in StAR gene expression, interrupting the signaling through this pathway at any step, by inhibiting COX2 activity (Wang et al., 2005; Wang et al., 2003b) or TBXAS activity (Wang et al., 2008) or blocking the TBX A2 receptors (Pandey et al., 2009), reduced the threshold, with sub-threshold levels of cAMP being able to induce maximal levels of StAR protein and steroidogenesis. These studies indicated that the signaling through COX2-TBXAS-TBX A2-receptor pathway plays an important role in regulating sensitivity of Leydig cells to LH or cAMP stimulation. Therefore, when COX2 increases in Leydig cell aging, the increase in COX2 enhances the negative signaling through this pathway, which in turn reduces the sensitivity of Leydig cells and inhibits the LH-induced StAR gene expression and testosterone biosynthesis.

4. Flavonoid intervention in the COX2-dependent inhibition of StAR gene expression and testosterone biosynthesis

To study the possibility of delaying the decline in blood testosterone by intervention in the mechanism, aged rats were fed with increasing concentrations of a selective COX2 inhibitor mixed in their diet. After 30 days, StAR protein in their Leydig cells increased in a concentration-dependent manner. The blood testosterone concentrations increased up to 120% over control (Wang et al., 2005). The studies suggest a possibility of delaying the age-related declines in StAR protein and testosterone using COX2 inhibitors. However, long-term application of the COX2 inhibitors currently used in the clinical practice is limited by their side effects. Therefore, alternative approaches are needed for the health of aging males. In the recent years, steroidogenic effects of natural flavonoids have been studied with Leydig cells. Flavonoids are a group of the polyphenolic compounds that are widely distributed in various food and food supplements, especially in fruits and vegetables. Previous studies have reported the activities of flavonoids in anti-inflammation, anti-cancer, and anti-oxidation (Cardenas et al., 2006; Chen et al., 1990; Ferrandiz & Alcaraz, 1991). One of the important mechanisms for these activities is the inhibition of COX2 expression and blocking the COX2-dependent signaling by flavonoids, which enables flavonoids to enhance StAR gene expression and testosterone biosynthesis in Leydig cells. A group of flavonoids

has been identified, including chrysin, apigenin, luteolin, and quercetin (Fig. 1), to be able to enhance StAR gene expression and steroidogenesis in Leydig cells by blocking the COX2-dependent signaling.

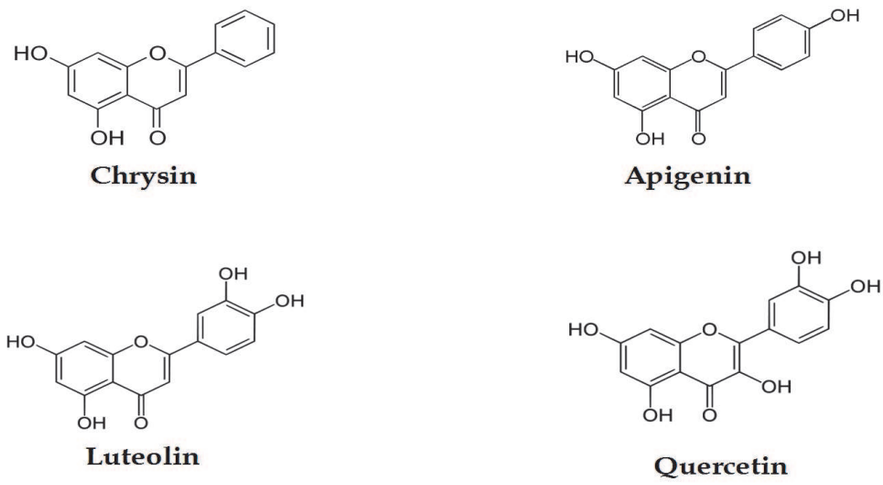


Fig. 1. Chemical structures of the flavonoids used in the experiments to enhance StAR gene expression and steroidogenesis in Leydig cells

4.1 Chrysin

Chrysin is present in plants (Williams et al., 1997), honey and propolis (Gambelunghe et al., 2003; Jiang et al., 2008; Kassim et al., 2010). It blocks the COX2-dependent signaling principally by inhibition of COX2 expression. The reduction of COX2 by chrysin is due to its inhibitory effects on the activities of several transcription factors that regulate COX2 gene transcription in different tissues. Chrysin is able to inhibit the activity of NF-kB and reduce COX2 promoter activity (Ha et al., 2010; Li et al., 2010). In macrophages, chrysin suppressed lipopolysaccharide (LPS)-induced COX2 expression by inhibiting activity of nuclear factor for IL-6 (NF-IL6) (Woo et al., 2005). NF-IL6 is a member of the C/EBP family that binds to human COX2 promoter region at -124/-132 and induces COX-2 expression. Another member of C/EBP family is C/EBPβ that is activated by c-Jun N terminal kinase (JNK) (Cho et al., 2003). Chrysin inhibited JNK activation and reduced LPS-induced COX2 expression (Ha et al., 2010). In addition, the cytokines, such as IL-1β and TNF-α, are able to induce COX2 expression (Chen et al., 2001a; Ishikawa et al., 2005). It was observed that chrysin significantly reduced the LPS-released IL-1β and TNF-α (Ha et al., 2010; Romier et al., 2008). In addition to the inhibition of COX2 gene expression, chrysin and its derivatives are able to inhibit COX2 activity. A group of chrysin derivatives was synthesized by modification of its molecular structure. The modification significantly improves the activities of the derivatives in inhibiting COX2. Some of the chrysin derivatives are able to bind to COX2 molecule and selectively inhibit COX2 activity (Cho et al., 2004; Dao et al., 2004).

Chrysin has been described as a testosterone-increasing agent that blocks further testosterone metabolism by inhibiting aromatase activity (Dhawan et al., 2002; Kellis & Vickery, 1984). This testosterone-increasing activity of chrysin was confirmed in the recent studies with Leydig cells isolated from mouse and MA-10 mouse Leydig cell line, with the steroid hormone productions of the Leydig cells being significantly enhanced by chrysin

(Jana et al., 2008). However, it failed to induce the significant increase in steroid hormone when MA-10 Leydig cells were co-incubated with 22(R)hydroxycholesterol, suggesting that this flavonoid increase steroidogenesis mainly by improving the cholesterol transfer to the mitochondrial inner membrane. This was proved by the results from Western blot analyses that showed a significant increase in StAR protein in the Leydig cells treated with chrysin. The observations were corroborated by the significant increases in StAR promoter activity and StAR mRNA levels in the cells, indicating that chrysin acted on StAR promoter and enhanced StAR gene transcription. The transcription factors that act on StAR promoter were examined in the Leydig cells incubated with chrysin. It was found that this flavonoid significantly reduced the transcriptional repressor, DAX-1 protein (Jana et al., 2008). As DAX-1 protein and DAX-1 mRNA were reduced, StAR protein and steroid hormone production dramatically increased. Similar to the effect of COX2 inhibitors, chrysin did not affect PKA activity. In the absence of cAMP, this flavonoid alone was not able to increase StAR protein and steroidogenesis. However, in the presence of 10 μm chrysin, sensitivity of Leydig cells dramatically increased, with sub-threshold levels of cAMP being able to induce maximal levels of StAR protein and steroidogenesis. The studies indicated that chrysin enhanced steroidogenesis in Leydig cells mainly by enhancing cAMP-induced StAR gene expression.

4.2 Apigenin

Apigenin is present in various plants at different concentrations (Miean & Mohamed, 2001). It was found in parsley at high level (Nielsen et al., 1999). This flavonoid blocks COX2-dependent signaling at two separated steps, by inhibiting COX2 expression and blocking the TBX A2 receptors. It inhibits COX2 expression at the levels of transcription and translation. At the transcriptional level, apigenin inhibits activities of the transcription factors that are important for COX2 gene expression. Previous studies reported that incubation of mouse macrophages with apigenin reduced COX2 expression by inhibition of NF-kB activation, through a mechanism involving the apigenin-reduced degradation of the inhibitor kB (IkB) (Liang et al., 1999). This observation was enhanced by the study on the apigenin-reduced COX2 expression, in which apigenin-inhibited IkBα degradation resulted in reductions in NF-kB-binding to the promoter DNA and the TNF-α-induced COX2 expression (Shukla & Gupta, 2004). In addition, apigenin is able to inhibit the activation of MAPK. It is known that MAPK activation is essential for the activities of several transcription factors, such as CREB, NF-kB, and C/EBP, that induce COX2 expression in various tissues (Chun & Surh, 2004; Tsatsanis et al., 2006). The inhibitory effects of apigenin on MAPK activation significantly reduced COX2 gene transcription (Ha et al., 2008; Yi Lau & Leung, 2010). At the translational level, apigenin increased the localization to cytoplasm of two proteins, HuR and T-cell-restricted intracellular antigen 1-related protein (TIAR). HuR and TIAR then bound to the AU-rich elements in the 3'-untranslated region of COX2 mRNA and inhibited COX2 translation (Tong et al., 2007). In addition to its inhibitory effects on COX2 expression, apigenin acts as a natural antagonist of the TBX A2 receptors. Binding of apigenin to the receptors blocked the signaling through COX2-TBXAS-TBX A2-receptors pathway (Guerrero et al., 2007; Navarro-Nunez et al., 2008).

It was reported that blocking the signaling at any step of the COX2-TBXAS-TBX A2-receptors pathway significantly increased StAR gene expression and testosterone biosynthesis in Leydig cells (Pandey et al., 2009). Therefore, the effects of apigenin on steroidogenesis were studied using MA-10 mouse Leydig cell line and Leydig cells isolated

from mouse. The binding of apigenin to the TBX A2 receptors was examined. The binding competition between apigenin and the selective receptor antagonist ^{3}H-SQ29548 indicated that this flavonoid specifically blocked the TBX A2 receptors on MA-10 mouse Leydig cells. Blocking the receptors with apigenin significantly increased StAR protein and testosterone production in mouse Leydig cells (Li et al., 2011). In MA-10 Leydig cells, apigenin induced concentration-dependent increases in StAR promoter activity, StAR mRNA, StAR protein and steroid hormone production. Further study indicated that this flavonoid enhanced StAR gene transcription by reduction of the transcriptional repressor, DAX-1 protein. While the mechanism for the reduction of DAX-1 protein in apigenin-treated cells is unknown, the results from the study suggest that minimal levels of PKA and protein kinase C (PKC) activities are essential for the effects of apigenin on DAX-1, StAR and steroidogenesis in Leydig cells (Li et al., 2011).

4.3 Luteolin

Luteolin is found in fruits and vegetables (Harnly et al., 2006). It was detected in bird chili (1035.0 mg/kg, in dry weight), belimbi leaves (464.5 mg/kg), onion leaves (391.0 mg/kg), belimbi fruit (202.0 mg/kg), dried asam gelugur (107.5 mg/kg), local celery (80.5 mg/kg), broccoli (74.5 mg/kg), carrot (37.5 mg/kg), limau purut leaves (30.5 mg/kg), French bean (11.0 mg/kg), and white radish (9.0 mg/kg) (Miean & Mohamed, 2001). Similar to the effects of apigenin, luteolin is able to inhibit COX2 expression and block the TBX A2 receptors. It was reported that luteolin reduced COX2 expression by inhibiting NF-kB activity (Chen et al., 2007a; Gutierrez-Venegas et al., 2006; Kim & Jobin, 2005), through the mechanism involving the luteolin-reduced IkB kinase (IKK) activity, IkB degradation, nuclear translocation of NF-kB p65 subunit, and NF-kB-binding to the promoter DNA. Luteolin may reduce COX2 expression by inhibiting activity of MAPK and subsequently reducing the activities of the transcription factors that are essential for COX2 transcription (Choi & Lee, 2010; Gutierrez-Venegas et al., 2006). It is known that COX2 expression is up-regulated by inflammatory cytokines, such as IL-1β and TNF-α. Luteolin was reported being able to reduce these cytokines, which may contribute to its inhibitory effect on COX2 expression (Wu et al., 2009). In addition, luteolin is also a natural antagonist of the TBX A2 receptors. It blocked the COX2-dependent signaling through COX2-TBXAS-TBX A2-receptor pathway by binding to the receptors (Guerrero et al., 2005; Guerrero et al., 2007).

The inhibitory effect of luteolin on COX2-dependent signaling suggests a possibility of using this flavonoid to enhance StAR gene expression and steroidogenesis in Leydig cells. This possibility was examined by the experiments with MA-10 mouse Leydig cells cultured for 6 hours in the medium containing increasing concentrations of luteolin and a cAMP analog (dbcAMP). StAR and DAX-1 proteins, steroid production, StAR mRNA and StAR promoter activity were analyzed as described in the previous study (Jana et al., 2008). As shown in Fig. 2A, the treatments with luteolin induced a concentration-dependent increase in steroid hormone production. Progesterone concentrations in culture medium were increased from 4.9 to 124.7ng/ml, as the levels of luteolin in the culture were increased from 0 to 12 μM. In the presence of 22(R)hydroxycholesterol(22R), there was no significant difference in steroid production among the treatments, suggesting that luteolin increase steroidogenesis by improving mitochondrial cholesterol transfer in Leydig cells. The results were enhanced by the luteolin-increased StAR protein expression. Similar increases were observed in the analyses of StAR promoter activity and StAR mRNA levels (Fig. 2B), indicating a regulatory effect of luteolin on StAR gene transcription. In addition, a synergistic interaction between

luteolin and cAMP was observed (Fig. 2C), in which luteolin reduced the threshold of cAMP-induced StAR gene expression and increased the sensitivity of Leydig cells to cAMP stimulation. Further study indicated that luteolin enhanced StAR gene transcription by inhibiting DAX-1 expression (Fig. 2D), similar to the observation with apigenin.

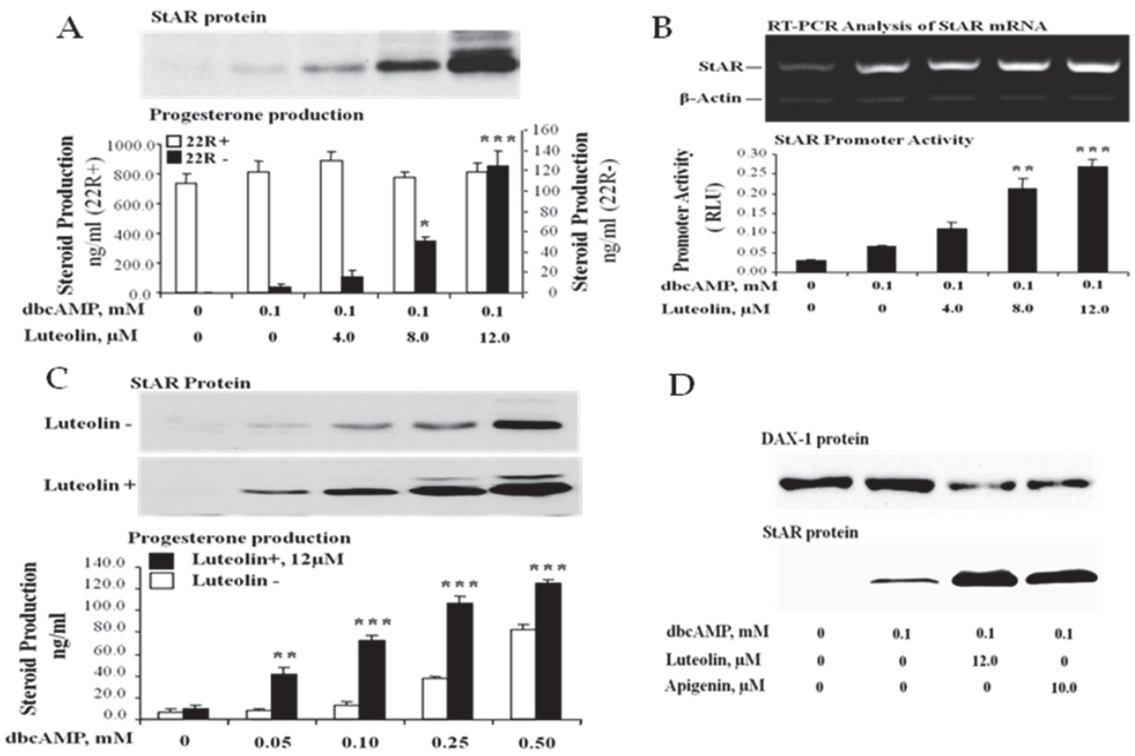


Fig. 2. Effect of luteolin on StAR gene expression and steroidogenesis in MA-10 mouse Leydig cells. MA-10 cells were incubated with luteolin for 6 hours, and then collected for analyses: A, StAR protein and steroidogenesis; B, StAR gene transcription; C, Interaction between luteolin and cAMP; D, Reduction in DAX-1 protein.

4.4 Quercetin

Quercetin was reported as one of the major flavonoids in the plants (Miean & Mohamed, 2001), especially in onions (Slimestad et al., 2007). It blocks the COX2-dependent signaling by inhibiting COX2 expression and TBX A2 production. Similar to other flavonoids, quercetin depresses COX2 expression by inhibiting activities of NF-kB. It was observed that this flavonoid reduced the activity of NF-kB by inhibiting IKK/IκB signaling cascade (Garcia-Mediavilla et al., 2007), which in turn reduced NF-kB nuclear translocation, its binding to promoter, and COX2 expression (Crespo et al., 2008; Puangpraphant & de Mejia, 2009). Another signaling pathway regulating COX2 expression is PI3K-Akt pathway. This signaling pathway may activate CREB (Alique et al., 2011) or NF-kB (Yang et al., 2009), and induce COX2 expression. A recent study found that quercetin directly bound with PI3K to inhibit PI3K activity and Akt phosphorylation, resulting in a reduction in COX2 expression (Lee et al., 2010). In the LPS-induced COX2 expression, the members of MAPK, including extracellular signal related kinase (ERK), p38 and JNK, are involved in the LPS-induced

signaling. While LPS activates the MAPKs, quercetin inhibited the activation of each of them, and subsequently reduced IκB phosphorylation and COX2 expression (Gutierrez-Venegas et al., 2007). The observations were enhanced by the study on the inhibitory effects of quercetin on ROS-induced MAPK activation and COX2 expression (Huang et al., 2006). The inhibition of MAPKs by quercetin was associated with the reductions in inflammatory cytokines, such as IL-1β and TNF-α (Overman et al., 2011). In addition, quercetin is able to block the COX2-TBXAS-TBX A2-receptor signaling pathway by inhibition of TBX A2 formation (Garcia-Saura et al., 2005; Sheu et al., 2004).

The steroidogenic effect of quercetin was studied with MA-10 mouse Leydig cells. It was reported that quercetin increased StAR mRNA levels, StAR promoter activity and steroid hormone production (Chen et al., 2007c). The observations were corroborated by the results shown in Fig. 3., which indicated the quercetin-enhanced StAR gene transcription and translation in MA-10 cells cultured for 6 hours (Fig. 3 A and B). Following these studies, the transcription factors in StAR gene transcription were examined. It was found that the incubation of MA-10 Leydig cells with quercetin reduced DAX-1 protein, similar to the effects of other flavonoids. The reduction in DAX-1 protein was associated with a dramatic increase in StAR protein (Fig. 3C), suggesting that quercetin enhance StAR gene expression by reduction of DAX-1 expression.

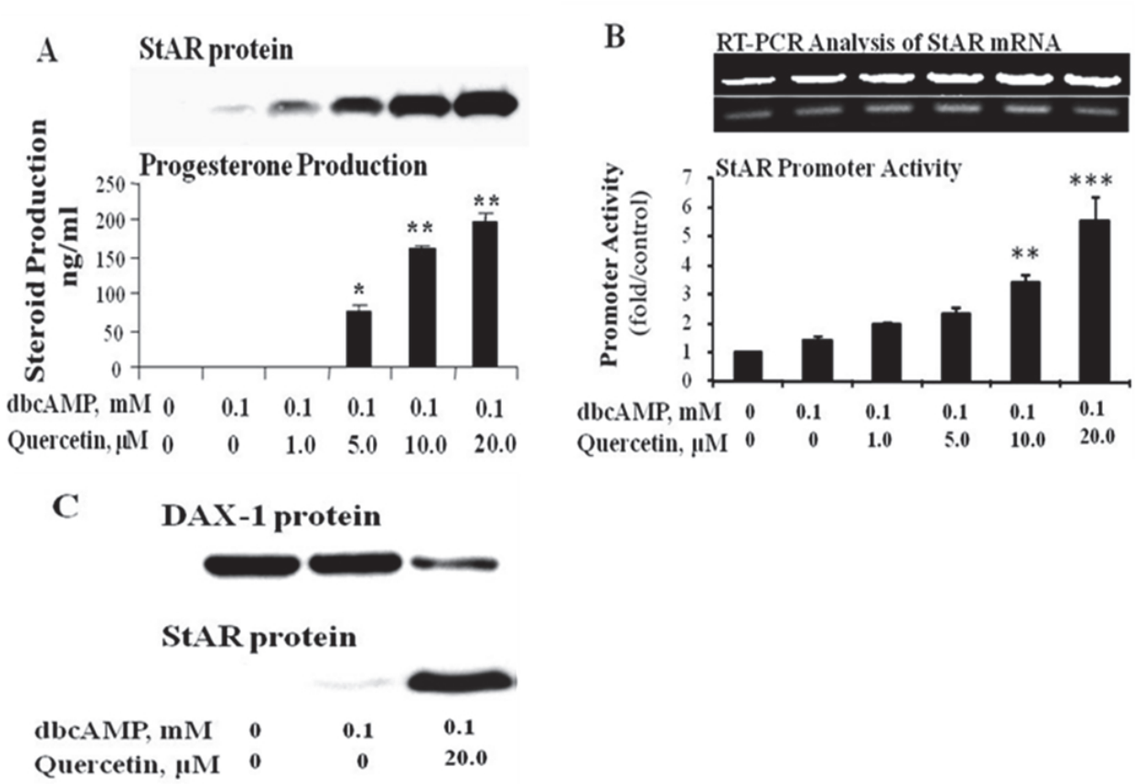


Fig. 3. Effect of quercetin on StAR gene expression and steroidogenesis in MA-10 mouse Leydig cells. MA-10 cells were incubated with quercetin for 6 hours, and then collected for analyses: A, StAR protein and steroidogenesis; B, StAR gene transcription; C, Reduction in DAX-1 protein

4.5 Potential application of natural flavonoids in delaying the declines in StAR gene expression and testosterone biosynthesis

The studies reviewed above suggest that flavonoids are potential resources for alternative medicine to delay the decline in StAR gene expression. Among the various compounds of natural flavonoids, many of them are able to reduce COX2 expression, such as Kaempferol (Garcia-Mediavilla et al., 2007), tectorigenin, tectoridin (Kim et al., 1999), wogonin (Chen et al., 2008) and Silibinin (Kim et al., 2009). Some of the flavonoids reduce the inflammatory factors that induce COX2 expression (Garcia-Lafuente et al., 2009; Khanna et al., 2007; Kim et al., 2004). Another group of flavonoids was reported as receptor antagonists that inhibit the COX2-dependent signaling by blocking the TBX A2-binding to the receptors (Navarro-Nunez et al., 2009). In addition to the COX2-dependent signaling, StAR gene expression is affected by the signaling through different pathways. For example, calcium influx through L-type calcium channels regulates the threshold of cAMP-induced StAR gene expression, so that blocking the L-type calcium channels reduced the threshold and enhanced StAR gene expression in Leydig cells (Pandey et al., 2010). There is a group of natural flavonoids that are able to block the L-type calcium channels, such as genistein (Belevych et al., 2002), daidzein (Yokoshiki et al., 1996), equol (Liew et al., 2003) and epigallocatechin-3-gallate (EGCG) (Kang et al., 2010). The steroidogenic effects of the flavonoids in this group were described (Yu et al., 2010). It is possible to identify different flavonoids that might enhance StAR gene expression and testosterone biosynthesis through different mechanisms.

The potential for application of natural flavonoids in delaying the decline in testosterone is supported by the animal studies on the flavonoid-enhanced testosterone production and reproductive function. A study with 2-year-old male rats reported that supplementation with chrysin improved their reproductive functions, with their sperm count, fertilization potential and litter size being increased when they were allowed to interact with female rats (Dhawan et al., 2002). It was also observed that lifetime exposure to the flavonoids, including daidzein, glycitein and genistein, increased serum and testicular testosterone concentrations of rats (McVey et al., 2004). The observation was enhanced by the study on the effects of catechins on testosterone biosynthesis in rats, in which injection with catechins or its derivatives increased testosterone in blood (Yu et al., 2010). A recent study reported the beneficial effects of quercetin in diabetic rats, with their sperm viability and motility being improved by the flavonoid. These effects of quercetin are associated with an increase in serum total testosterone (Khaki et al., 2010). In addition, some flavonoids in herbal medicine are able to enhance testosterone production in rats. For example, icariin, a flavonoid in the plants in *Epimedium* family, has been extracted from traditional Chinese medicine (Du et al., 2002). It was observed that supplementation of icariin to 15-month-old male rats significantly increased their blood testosterone levels (Zhang & Yang, 2006).

For application of natural flavonoids to improve the health of aging males, further studies, especially long-term animal studies and clinical studies, are needed. Two of the important considerations in the studies are mechanism and efficacy of the flavonoid-enhanced testosterone biosynthesis. The mechanisms reported for the inhibitory effects of flavonoids on COX2-dependent signaling are mostly based on the studies with the cells from various tissues. These mechanisms need to be verified with Leydig cells. In addition to the COX2-dependent signaling, other mechanisms might be involved in the flavonoid-enhanced StAR gene expression and testosterone production. Further studies on the mechanisms may improve the understanding of the steroidogenic effects of flavonoids. Regarding the efficacy, bioavailability is an important factor being discussed in the studies on flavonoids

(Setchell et al., 2001; Setchell et al., 2002). Specifically in the flavonoid-enhanced StAR gene expression and testosterone production, the following factors have been discussed recently: 1) Biphasic effects of flavonoids on StAR gene expression. As mentioned above, some of the flavonoids are able to block L-type calcium channels. It was reported that blocking L-type calcium channel produced biphasic effects on StAR gene expression in Leydig cells (Pandey et al., 2010). While these flavonoids might enhance StAR gene expression by reducing the transcriptional repressor DAX-1 protein, they might inhibit the positive effect of calcium-dependent signaling on StAR gene expression; 2) Inhibitory effects of flavonoids on steroidogenic enzymes. It was reported that some of the flavonoids are able to inhibit activities of the enzymes involved in testosterone biosynthesis (Figueiroa et al., 2009; Hu et al., 2010). These inhibitory effects may reduce the efficacy of flavonoid-enhanced testosterone production; 3) Levels of cAMP in Leydig cells. The levels of cAMP-PKA-phosphorylation are critical for the flavonoid-enhanced StAR gene expression in Leydig cells. As shown in Fig. 2C, in the absence of cAMP, flavonoid alone is not able to induce significant increases in StAR protein expression and steroid hormone production (Jana et al., 2008; Li et al., 2011). In the studies without exogenous cAMP, the endogenous cAMP becomes important for the steroidogenic effect of flavonoids. When the levels of endogenous cAMP or PKA activity in Leydig cells are not sufficient, flavonoid might not be able to induce significant increase in StAR gene expression. Therefore, minimal level of cAMP or PKA activity should be considered for the flavonoid-enhanced StAR gene expression and testosterone biosynthesis.

5. Summary

In summary, LH-stimulation of Leydig cells induces both positive and negative signals in the regulation of StAR gene expression and testosterone biosynthesis. In the aging process, the increase in COX2 enhances the negative signaling, resulting in the declines in StAR gene expression and testosterone biosynthesis in Leydig cells. It was found that some of the flavonoids are able to block the COX2-dependent signaling and enhance StAR gene expression. While further investigations are needed, the studies suggest a potential for application of the natural flavonoids in delaying the age-related declines in StAR gene expression and testosterone biosynthesis.

6. Acknowledgment

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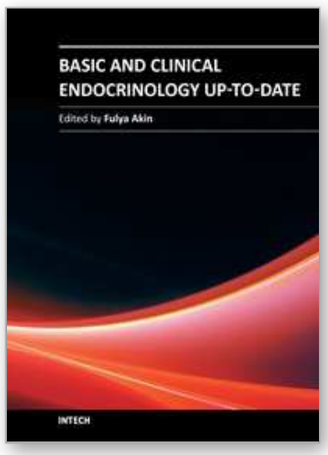
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This book provides the most up-to-date information on the basic and clinical aspects of endocrinology. It offers both researchers and clinicians experts, gold-standard analysis of endocrine research and translation into the treatment of diseases such as insulinoma, endocrine disease in pregnancy and steroid induced osteoporosis. Investigates both the endocrine functions of the kidneys and how the kidney acts as a target for hormones from other organ systems. Presents a uniquely comprehensive look at all aspects of endocrine changes in pregnancy and cardiovascular effects of androgens.

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