Effect of Berberine on Testosterone secretion from Testicular TM3 cell lines in hypoxia condition.

Yi – Jing Chen¹, Ju-Wen Yu¹, Hea-Wen Tzou¹, Yuan-Hao Chang¹, Ya-Wen Jeng² and Shyi-Wu Wang²*

¹Graduate Institute of Biomedical Sciences, ²Department of Physiology and Pharmacology, College of Medicine, Chang-Gung University, Kweisan, Taoyuan, Taiwan, ROC

Key words: hypoxia, berberine, Leydig TM3 cells, testosterone

*Correspondence: Shyi-Wu Wang, Ph. D.
Department of Physiology and Pharmacology
Chang Gung University
Taoyuan 33333, Taiwan,
Republic of China.

Tel No.: 886-3-211-8800 EXT 5253
Fax No.: 886-3-3288448
Abstract

Berberine is an isoquinoline alkaloid isolated from herb plants, such as *Cortex phellodendri* (Huangbai) and *Rhizoma coptidis* (Huanglian). Huanglian and Huangbai have used as “heat-removing” agents. In addition, berberine has been reported to have anti-inflammatory effects both in vivo and in vitro. Hypoxia is a major stimulator to cause the pathogenesis of many diseases such as heart disease, neuron death, cardiovascular diseases, and cancer. Tumors hypoxia has been indicated to be strongly associated with tumor malignant progression, propagation, and resistance to therapy. Therefore, hypoxia has become a central issue in tumor physiology and cancer treatment. However, the effect of berberine on male reproduction in hypoxia condition is still not known. The purpose of this study is to investigate the effect of berberine in hypoxia condition on testosterone secretion from TM3 cells, a kind of rat Leydig cell lines. In 12-well plate, TM3 cells (4×10^4 /ml) were incubated with berberine (10, 20, 50 μg/ml) under normoxia (21% O_2) or hypoxia (1% O_2) for 1 hour and treated with or without hCG (0.5 IU/ml) for 16 hours. Media were collected later, centrifuged, and stored in –20°C for testosterone EIA. Meanwhile, different activators/inhibitors of signal transduction with berberine and hCG were added into the media. Stimulatory effect of berberine on testosterone release from TM3 cells was observed under hypoxia condition. Significant inhibitory effect of nifedipine (L-type calcium channel blocker), U73122 (PLC inhibitor), and helenaline (NF-κB inhibitor) were found in TM3 cells under hypoxia condition. In contrast, AG490 (JAK-2 inhibitor), forskolin (activator of adenylyl cyclase), SQ22536 (adenylyl cyclase inhibitor), and GF109203X (PKC inhibitor) did not affect the testosterone secretion from TM3 cells under hypoxia condition. Our results showed that, under hypoxia condition, the stimulatory effect of berberine on testosterone secretion from Leydig TM3 cells maybe mediated through phospholipase C, calcium channel, and NF-κB pathways.
Introduction

Berberine is an isoquinoline alkaloid (Zhou and Mineshita, 2000) existed in Hydrastis Canadensis (goldenseal), Cortex phellodendri (Huangbai), and Rhizoma coptidis (Huanglian) (Ikram 1975). Extracts from these plant root or skin are used for disease treatment, e.g. lumbago, rheumatism, and fever (Yesilada and Kupeli 2002). Huanglian has been used as a herb in India and China for more than 3000 years (Shirwaikar, Shirwaikar et al. 2006). As a herb, huanglian has been employed as prophylactic drug, and for GI disorder (Lin, Chung et al. 2005). Studies indicated that berberine poses the inhibitory effect on cyclooxygenase-2 activity and lipoygenase (Misik, et al., 1995), interleukin-8 increase in inflammatory enteric disease (Nielsen, et al., 1987), and colitis induced by trinitrobenzene sulfonic acid (Zhou and Mineshita, 2000). Also, the edema in hind paw in mice induced by serotonin can be inhibited by berberine in vivo (Kupeli, et al., 2002). Moreover, studies showed that berberine can be used in treatment hepatic injury (Hwang, et al., 2002), thyroxine induced ventricular hypertrophy (Yang, et al., 2006), hypercholesterolemia (Kong, et al., 2004), decreased bone mineral density (Wu, et al., 1999; Li, et al., 2006), parasite infection (Subbaiah and Amin, 1967; Stermitz, et al., 2000; Hawrelak J, 2003; Kong, et al., 2004), erectile-dysfunction (Chiou, et al., 1998; Drewes, et al., 2003), tumor metastasis (Hoshi, et al., 1976; Fukuda, et al., 1999; Marinova, et al., 2000; Mitani, et al., 2001; Peng, et al., 2006; Yang, et al., 2006).

Studies showed that hypoxia decreased thyrotrrophs and thyroid cells (Gonsly, 1985), promoted glucocorticoid (Raff, et al., 2003) and erythropoietin secretion (Wang, et al., 1996), inhibited aldosterone secretion (Raff, et al., 1997 ; Raff and Bruder, 2006), disturbed the hypothalamic-pituitary-gonadal function in male (Wang AC, 1990), lowered LH/FSH (Semple et al., 1981; Farias et al., 2008) and testosterone secretion(Semple et al., 1984). Also, Obstructive sleep apnea is considered to relate to intermittent hypoxia condition. In these patients, low testosterone concentration is a common syndrome (Santamaria, Prior, and Fleetham. 1988; Hu et al., 1998; Luboshitzky et al., 2002; Gambineri, Pelusi, and Pasquali. 2003; Luboshitzky et al., 2003; Luboshitzky et al., 2005; Zhuravlev et al., 2009). This hypogonadism has been related to age and obesity (Gambineri, Pelusi, and Pasquali. 2003; Luboshitzky et al., 2002; Luboshitzky et al., 2005). However, Coste et al. (2006) indicated that there is no significant relationship between hypoxia and hypogonadism. In addition, our lab has demonstrated that hypoxia stimulated testosterone secretion in Leydig TM3 cells (Hwang et al., 2007) and increased testosterone secretion in TM3 cells was found in intermittent hypoxia (Hwang et al., 2009).

In the present study, mouse Leydig cells were used to investigate the effects of berberine on (1) testosterone secretion, (2) signal transduction, and (3) the involvement of basic regulatory pathways in regulation of HIF-1α, VEGF, and ERK1/2 expression under normoxia and hypoxia condition.
Materials and Methods

Antibodies and reagents.

Human chorionic gonadotropin (hCG) and mouse VEGF were purchased from Sigma (St. Louis, MO). PD-98059 was purchased from Tocris Cookson (Westwoods Business, Park Ellisville, MO). Anti-VEGF (1:200 dilution), anti-phospho (p)-extracellular signal-regulated kinases 1 and 2 (ERK1/2; 1:1,000 dilution), anti-β-actin (1:8,000 dilution), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:500 dilution) were purchased from Santa Cruz (Watsonville, CA). The horseradish peroxidase-conjugated IgG, goat anti-rabbit IgG (1:6,000 dilution), and goat anti-mouse IgG (1:8,000 dilution) were purchased from ICN Pharmaceuticals (Aurora, OH).

Cell culture.

TM3 Leydig cells, a nontumorogenic cell line derived from mouse testis, were obtained from the Culture Collection and Research Center (Food Industry Research and Development Institute, Taiwan, Republic of China). This cell line responds to LH by increasing testosterone production and secretion through mechanisms similar to those encountered in freshly isolated cells. Cells were cultured in 75-cm² flasks (Falcon, Franklin Lakes, NJ) in a 1:1 mixture of Ham’s F-12 and DMEM (Sigma) that contained 15 mM HEPES, 0.12% NaHCO₃ supplemented with 0.45% glucose, 5% horse serum, 2.5% FCS (Kibbutz Beit, Haemek, Israel), and 100 IU/ml potassium penicillin G + 100 μg/ml streptomycin sulfate (Sigma). Cells were cultured at 37°C in either a humidified atmosphere of normoxic conditions (95% air-5% CO₂) or in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) and flushed with hypoxic gas (95% N₂-5% CO₂).

ELISA of Testosterone

Concentrations of testosterone in collected media were determined by
ELISA (Enzyme-linked Immunosorbent Assay). Briefly, 0.1 ml of capture antibodies (R & D systems, Minneapolis, MN, USA) were coated on the polystyrene microtiter plates (NUNC, U16 Maxisorp type, Rochester, NY, USA) and incubated at room temperature overnight. The plates were blocked the next day and then incubated for 1 h. Then, 0.1 ml standard/sample was added and incubated for 2 h. After washing 3 times, 0.1 ml of detection antibody (R & D systems) was applied for 2 h. One hundred microliters streptavidin horseradish peroxidase (R & D systems) and then 0.1 ml tetramethylbenzidine substrate (Clinical Science Products Inc., Mansfield, MA, USA) followed this incubation. The reaction was stopped using 2 N sulfuric acid and optical density (OD) reading was taken at 450 nm (BioTek, Winooski, VT, USA). All samples were run in duplicates. The results were expressed as concentration of testosterone (pg/ml) read from standard curves. The detection range, sensitivity, the intraassay and interassay coefficients of variation are: 7.8-500 pg/ml, 6 pg, 4.4%, and 7.7%, respectively.

**Effect of hypoxia on the expressions of ERK1/2.**

Mouse TM3 Leydig cells (106 cells/10 ml) were seeded in 10-cm dishes and then incubated with or without hCG at 1 IU/ml for 1 or 16 h in a normoxic or hypoxic condition. At the end of incubation, cytoplasmic proteins were extracted from the cells and used to determine the expression of VEGF, HIF-1α, p-ERK1/2, and p-p38 through Western blot.

**Statistical analysis.**

The data were expressed as means ± SE. The treatment means were tested for homogeneity using ANOVA, and the difference between specific means was tested for significance using Duncan’s multiple-range test or Student’s t-test (Steel & Torrie, 1960). A difference between two means was considered to be statistically significant when the P value was <0.05.
**Results**

*Effect of glycyrrhizin and berberine on testosterone secretion under normoxia*

Significant increases of testosterone were found in TM3 cells treated with berberine (10~50 μg/ml) or hCG (0.5 IU/ml) (Fig. 1).

*Signal pathways in TM3 after berberine treatment*

Increased testosterone secretion were found in TM3 cells treated AG490 (Fig 3), Nifedipine (Fig 4), SQ22536 (Fig 5), U73122 (Fig 6), GF109203X (Fig 7), Helenaline (Fig 8), and berberine.

No change in testosterone secretion was found in TM3 cells after forskolin (Fig. 2). Also, these activators/inhibitors had no effect on testosterone secretion from TM3 cells treated with berberine and hCG (Fig. 9).

*Effect of berberine on testosterone from TM3 under hypoxia condition*

Significant increase of hCG on testosterone secretion from TM3 under hypoxia were found at 0.5 IU/ml (Fig. 10A) and 16 h (Fig. 10B). Increased testosterone secretion were observed in TM3 treated with berberine (10-20 μg/ml) only or with hCG (0.5 IU/ml) (Fig. 11A).

*Signal pathway in TM3 after berberine treatment under hypoxia condition*

No difference in testosterone concentration was found between berberine only and berberine + forskolin (10⁻⁶ M) (Fig. 11B), AG490 (10⁻⁶ M) (Fig. 12A) treatment under hypoxia condition. Decreased testosterone was observed in TM3 with berberine + nifedipine (Fig. 12B), U73122 (Fig. 13B), helenaline (Fig. 14B) compared to berberine treatment only in hypoxia. However, increased testosterone was found in TM3 with berberine + SQ22536 compared to berberine only (Fig. 13A). Decreased media testosterone was found only in 10 μg/ml berberine + GF109203X compared to berberine only in hypoxia (Fig. 14A).

*Effect of berberine on HIF-1α, VEGF, and ERK1/2 expression in TM3 under*
**hypoxia condition**

No difference was found in VEGF in expression in TM3 between berberine only or with hCG under hypoxia condition (Fig. 15A). Augmented HIF-1α expression was found in TM3 with berberine only. In contrast, lower expression of HIF-1α was observed after berberine and hCG treatment (Fig. 15B). Enhanced p-ERK1/2 expression was found in TM3 with berberine only or berberine + hCG treatment (Fig. 16A). Also, stimulated p-p38 expression with berberine only or berberine + hCG treatment (Fig. 16B)
Discussion

In the present studies, we found that: (1) the stimulatory effect of berberine on testosterone secretion from TM3 might be through calcium ion channel, adenylyl cyclase, phospholipase C, protein kinase C, and NF-κB; (2) under hypoxia condition, the stimulatory effect of berberine on TM3 cells is part through adenylyl cyclase, protein kinase C, phospholipase C, NF-κB, HIF-1α, ERK1/2, and p38.

Previous studies have indicated that regulation of MAPK is associated with the action of berberine in tumors or cells. Berberine inhibits the expression of inflammatory cytokines in ARPE-19 cells, a human retinal pigment epithelial cell line, through down-regulation of the ERK1/2, JNK, and p38 pathways (Wang, et al., 2012). Yan et al. (2012) showed that berberine promotes recovery of dextran sulfate sodium (DSS)-induced colitis and decreases proinflammatory cytokine production through MAPK. Also, in human colonic carcinoma cells, berberine reduces cAMP-induced chloride secretion, which is mediated by stimulation of ERK1/2 and p-38 (Alzamora, et al., 2011). Berberine hydrochloride attenuates LPS-induced COX-2 expression and p38 activation in small intestinal mucosa cells (Feng, et al., 2011). In addition, through the activation of ERK1/2 and p38, berberine chloride induced the anti-Leishmanial activity in macrophages (Saha, et al., 2011). In lung tumor, administration of berberine inhibits cell cycle progression and tumorgenesis through Akt, CREB and MAPK pathways (James, et al., 2011). Zhang, et al. (2011) demonstrated berberine moderates glucose and lipid metabolism through the activation of AMPK, p38, JNK and PPARα. In our study, we found that berberine, in normoxia or hypoxia condition could regulate testosterone secretion from TM3 cells through the activation of either ERK1/2, JNK, or p38. This finding is in agreement with these previous studies.

In conclusion, our results demonstrate (1) glycyrrhizin stimulated testosterone
secretion from TM3 cells at least in part through the adenylyl cyclase, JAK-2, calcium ion channel, and protein kinase C; (2) the stimulatory effect of berberine on testosterone secretion from TM3 is via calcium ion channel, adenylyl cyclase, phospholipase C, protein kinase C, and NF-κB; (3) under hypoxia condition, glycyrrhizin stimulates testosterone secretion from TM3 cells through JAK-2, calcium ion channel, HIF-1α, and ERK1/2; (4) the stimulatory effect of berberine on TM3 cells is part through adenylyl cyclase, protein kinase C, phospholipase C, NF-κB, HIF-1α, ERK1/2, and p38.
Acknowledgments

This study was supported by the grant (CMRPD10041 and CMRPD10042) from Chang-Gung Memorial Hospital, Taipei, Taiwan, ROC.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.
References


Alzamora R, O'Mahony F, Ko WH, Yip TW, Carter D, Irnaten M, and Harvey BJ. Berberine Reduces cAMP-Induced Chloride Secretion in T84 Human Colonic Carcinoma Cells through Inhibition of Basolateral KCNQ1 Channels. Front Physiol, 2011; 2: 33.


Imanishi, N., Kawai, H., Hayashi, Y., Yatsunami, K., and Ichikawa, A. Effects of


Wang AC. [Changes in hypothalamic-pituitary-gonadal function in male patients with
acute attack of chronic cor pulmonale]. Zhonghua Jie He He Hu Xi Za Zhi 1990 13: 102–105,


Figure 1. Effect of berberine on testosterone secretion from TM3 cells treated with or without hCG (0.5 IU/ml) under normoxia condition.

Figure 2. Effect of berberine on testosterone secretion from TM3 treated with or without forskolin (10^-6 M; adenylyl cyclase activator). n=4.
Figure 3. Effect of berberine on testosterone secretion from TM3 treated with or without AG490 (10⁻⁶ M; JAK-2 tyrosine kinase inhibitor). n=4.

Figure 4. Effect of berberine on testosterone secretion from TM3 treated with or without nifedipine (10⁻⁴ M; L-type calcium channel inhibitor). n=4.
Figure 5. Effect of berberine on testosterone secretion from TM3 treated with or without SQ22536 (10^{-5} M; adenylyl cyclase inhibitor). n=4.

Figure 6. Effect of berberine on testosterone secretion from TM3 treated with or without U73122 (10^{-4} M; phospholipase C inhibitor). n=4.
Figure 7. Effect of berberine on testosterone secretion from TM3 treated with or without GF109203X (10^{-6} M; protein kinase C inhibitor). n=4.

Figure 8. Effect of berberine on testosterone secretion from TM3 treated with or without helenaline (10^{-6} M; NF-κB inhibitor). n=4.
Figure 9. Effect berberine (50 µg/ml) on testosterone secretion from hCG-treated TM3 cells with or without activators/inhibitors.
Figure 10. Effect of hCG on testosterone secretion from TM3 under hypoxia condition in different dosage of hCG(A) and time course (B).
Figure 11. Effect of berberine on testosterone secretion from TM3 treated with hCG (A) and forskolin (B) under hypoxia condition.
Figure 12. Effect of berberine on testosterone secretion from TM3 treated with hCG and (A) AG490 or (B) nifedipine under hypoxia condition.
Figure 13. Effect of berberine on testosterone secretion from TM3 treated with hCG and (A) SQ22536 or (B) U73122 under hypoxia condition.
A.

Figure 14. Effect of berberine on testosterone secretion from TM3 treated with hCG and (A) AG490 or (B) nifedipine under hypoxia condition.
Figure 15. Effect of berberine on (A) VEGF and (B) HIF-1α expression in TM3 treated with hCG (0.5 IU/ml) under hypoxia condition.
Figure 16. Effect of berberine on (A) ERK and (B) p38 expression in TM3 treated with hCG (0.5 IU/ml) under hypoxia condition.