European Journal of Obstetrics & Gynecology and Reproductive Biology 174 (2014) 123–127

Contents lists available at ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.elsevier.com/locate/ejogrb

Calcium concentration response to uterine ischemia: a comparison of uterine fibroid cells and adjacent normal myometrial cells

Weihong Yang, Zhongping Cheng *, Hong Dai

Department of Obstetrics and Gynecology, Yang-Pu Center Hospital, Shanghai, China

ARTICLE INFO

Article history:
Received 15 April 2013
Received in revised form 7 December 2013
Accepted 9 December 2013

Keywords:
Uterine artery occlusion
Fibroids
Apoptosis
Calcium release

ABSTRACT

Objective: Uterine artery occlusion by laparoscopy (UAOL) has been used for the treatment of uterine fibroids and beneficial effects to patients have been shown in clinical studies since 2000. Fibroid cells are more susceptible to apoptosis than myometrial cells under hypoxic conditions, but the molecular mechanisms underlying this effect remain unclear. The aim of this study was to investigate the role of intracellular calcium (Ca^{2+}) release mediated by Ca^{2+} channel inositol 1,4,5 trisphosphate receptor1 (IP3R1)/ryanodine receptor1 (RYR1) in the apoptosis of uterine fibroid cells under hypoxia.
Study design: We compared the expressions of IP3R1 and RYR1 in fibroid and surrounding myometrial tissue from 20 patients before UAOL. After 6 h treatment under hypoxia (1% O_2) with or without Ca^{2+} channel blockers (heparin or/and ruthenium red), the intracellular Ca^{2+} concentration, cytochrome c (Cytc) protein and cell apoptosis were determined.
Results: IP3R1 and RYR1 mRNA and protein levels were significantly higher in fibroid than in myometrial tissues. Under hypoxic conditions, Ca^{2+} concentration in fibroid cells was significantly higher than in myometrial cells (Ca^{2+}: 82.69 ± 16.92 nmol/L vs 46.14 ± 9.11 nmol/L, $P < 0.05$), and Cytc increased similarly in fibroid cells. These increases in Ca^{2+} concentration, Cytc and cell apoptosis were significantly reversed by calcium blocker in fibroid cells.
Conclusion: This study demonstrated that intracellular calcium release mediated by IP3R1/RYR1 could induce apoptosis in uterine fibroid cells under hypoxic conditions, and was responsible for the susceptibility to apoptosis of fibroid cells under UAOL.

1. Introduction

In recent years, uterine artery occlusion (UAO), which is an alternative to hysterectomy in the treatment of uterine fibroids, has made great progress in clinical practice [1–4]. The mechanism of UAO treatment, however, is still unknown. In 1994, Ravina et al. demonstrated that fibroids shrank in size and menorrhagia was diminished following bilateral injection of plastic particles into the uterine arteries [5]. To explain the observation that fibroid tissue died while normal myometrium survived, these authors postulated that the injected particles were preferentially delivered to the fibroid tissue. In 1999, Lee et al. demonstrated that similar results to uterine artery embolization (UAE) could be obtained by laparoscopically occluding the uterine arteries with metal clips [6]. In 2000, Burbank and Hutchins postulated that fibroid cells had greater sensitivity to hypoxia than normal myometrium and this was the mechanism behind UAE and UAO [7]. In 2009, Burbank reported that UAE and UAO reproduce the ischemia that occurs naturally throughout the uterus during childbirth [8]. Fibroid cells have been shown to be more sensitive to hot and cold [9]. Tissue plasminogen activator concentration is higher in myometrium than in fibroids [10]. The hypoxia-inducible gene, HIF-1, has been shown to be overexpressed in fibroid cells compared to myometrium [11]. Finally, apoptosis has been shown to be greater in fibroid cells following UAO [12,13]. The exact mechanism of apoptosis of fibroid cells, however, is still not fully discovered.

As is well known, intracellular free calcium (Ca^{2+}) is an important second messenger in the intrinsic cell apoptosis pathway. Overload of Ca^{2+} increases the permeability of mitochondrial permeability transition pores (MPTP), and then cytochrome c (Cytc) is released into the cytoplasm and initiates a downstream caspase cascade, ultimately leading to apoptosis [14]. Intracellular Ca^{2+} release is regulated by the calcium channel inositol 1,4,5 trisphosphate receptor (IP3R) and ryanodine receptor (RYR) in sarcoplasmic reticulum membrane, and the distribution of IP3R1 and RYR1 are relatively abundant in smooth muscle cells. We therefore speculated that the release of Ca^{2+} mediated by IP3R1/RYR1 may be related to the apoptosis of uterine fibroids

* Corresponding author. Tel.: +86 2165690520; fax: +86 2165676697.
E-mail addresses: mdcheng18@263.net, zpcheng_63@126.com (Z. Cheng).

0301-2115/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ejogrb.2013.12.013

under hypoxia. This study was performed to further investigate the different effects of hypoxia on fibroid and myometrial cells. We examined the concentrations of intracellular Ca^{2+} and the expression level of Cytc protein, and the expression of inositol 1,4,5 triphosphate receptor 1 (IP3R1) and ryanodine receptor 1 (RYR1) genes in fibroid cells and adjacent normal myometrial cells following 6 h of laboratory-induced hypoxia.

2. Materials and methods

2.1. Reagents

Trizol reagent was purchased from Invitrogen. cDNA synthesis kit was purchased from MBI Fermentas (Thermo Scientific). PCR kit was purchased from Takara Bio Inc. The primary antibody IP3R1 (ab5804), RYR1 (ab2868) and anti-cytochrome C antibody (ab13575) were purchased from Abcam (Cambridge, MA, USA). Protein quantification kit (BCA method) was purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

2.2. Tissue samples

Informed consent was obtained from all patients and this study was approved by the ethics committee of the Yand-Pu Hospital, Shanghai, China. Patients were randomly selected from our department between May 2011 and July 2011, and 20 uterine fibroid cases were studied. The mean age of patients was 43.6 ± 2.7 years. Fibroid and myometrial tissues were obtained by laparoscopy combined with myomectomy before patients were treated for symptomatic fibroid disease using the UAO technique as described previously [12]. Fresh tissue specimens 1.5 cm × 1.0 cm × 1.0 cm in size were then placed in sterilized frozen tubes, and stored at −80 °C. The diagnosis was confirmed by histological examination of the specimens removed.

2.3. Real time PCR

IP3R1 and RYR1 gene expressions in 20 randomly selected cases of uterine fibroid were measured by real-time PCR with the SYBR Green I method [14]. Total RNA was extracted with Trizol reagent from frozen tissue samples. cDNA was synthesized by reverse transcriptase at 37 °C for 1 h and 95 °C for 5 min. The following primer sets were used: IP3R1 forward primer: 5′-TATTCGGGTGCTGTCTGATG-3′, reverse primer: 5′-ACAATGCTGTGGGACTTGAG-3′, RYR1 forward primer: 5′-GACCGCCTAAATGTCTACAC-3′, reverse primer: 5′-ACGATTGCCACGGATTAGAG-3′, GAPDH was used as a reference gene, forward primer: 5′-GCACCGTCAAGGCTGAGAAC-3′, reverse primer: 5′-GCCTTCTCCATGGTGGTGAA-3′. PCR amplification was performed with the following amplification scheme: denaturation for 2 min at 50 °C, denaturation for 5 min at 95 °C, 40 cycles of denaturation for 15 s at 95 °C and 35 s at 60 °C, and carried out in ABIprism 7500 (Life Technologies). Gene expression was calculated as the relative gene expression (ΔCT).

2.4. Western blot

Protein was extracted with RIPA buffer containing protease inhibitors, and centrifuged at 12,000 × *g* for 15 min at 4 °C, and the supernatant protein was quantified and stored at −80 °C. Total protein was detected by the BCA method. Samples were boiled for 5 min, and 30 μg of total proteins per well were loaded for separation. After electrophoresis, proteins were transferred to a nitrocellulose membrane, blocked with TBST containing 3% BSA for 60 min at room temperature, then added primary antibody (anti-IP3R1, 1:1000; anti-RYR1, 1:1000; anti-Cytochrome C, 1:2000), and incubated at room temperature for 2 h. After washing, secondary antibody was incubated for 1 h at room temperature. Bands were developed by enhanced chemiluminescence (ECL) and scanned by the ImageQuant LAS4000 (GE healthcare), Image J software was used to analysis gray value of bands.

2.5. Primary cell culture and treatment

Fresh uterine myometrium tissue was isolated and cut into pieces in HBSS buffer containing penicillin and streptomycin. After adding 0.4% collagenase (containing DNase I 20 μg/ml), 0.05% trypsin 2 ml, the sample was shaken at 37 °C for 3 h for digestion. Following the digestion, cells were washed and seeded in culture flasks containing 10% FBS and 100 U/ml of penicillin and 100 μg/ml of streptavidin in DMEM-F12 medium. The adherent cells were fibroblasts within 20 min, and non-adherent cells were transferred to new flasks; after 3 cycles of 20 min adherence, the non-adherent cells were smooth muscle cells and attached overnight. The purity of both leiomyoma cells and smooth muscle cells was assessed by markers of CD90, FAP and α-actin. CD90 and FAP are markers of fibroblast, and α-actin is a marker of smooth muscle cells: the cells used were relatively pure as shown in supplementary material.

Cells were divided into four groups. Group A was treated with the IP3R blocker heparin (0.1 μM), group B was treated with the RYR blocker ruthenium red (1 μM), group C was treated with heparin (0.1 μM) and ruthenium red (1 μM), and group D was a vehicle group. Then, both uterine fibroid cells and smooth muscle cells were treated with compounds for 6 h under hypoxia (1% O_2), and cells cultured in normoxic condition were selected as a negative control in Annexin V and PI staining assay. Western blot was used to detect the expression of Cytc, and cell apoptosis was measured by flow cytometry (BD FASC) with Annexin V and PI staining, using a commercial kit (Kaiji Biotechnology, Nanjing, China).

2.6. Determination of intracellular Ca^{2+}

Intracellular Ca^{2+} level was determined according to the method of Mukherjee et al. [15] with slight modifications. Cells were collected after treatment, and incubated for 1 h after adding 5 μmol/L of Fluo-3-Am. Then, cells were centrifuged to remove excess dye, washed twice with non-calcium buffer, and finally diluted with a non-calcium buffer. Fluo-3-Am as a highly specific Ca^{2+} fluorescent indicator can sensitively reflect the changes of the intracellular free calcium concentration. Ten percent of Triton X-100 and 1 mmol/L of calcium chloride were added to the cell lysate, and after incubating for 5 min, F_{max} value was measured: twenty microliters of EDTA was added and incubated for 5 min followed by measuring F_{min} value. Ca^{2+} concentration was calculated as $K_d \times (F - F_{min})/(F_{max} - F)$. K_d is the dissociation constant of the fluorescent agent with Ca^{2+} (400 nmol/L), and F is the fluorescence intensity of cells measured.

2.7. Statistical methods

Data were expressed as mean ± SEM, Student's *t*-test was used to compare the difference of samples, and the difference was considered statistically significant at $P < 0.05$.

3. Results

3.1. Over-expression of IP3R1 and RYR1 in uterine fibroid tissue

This result showed that calcium channel proteins IP3R1 and RYR1 were significantly increased in uterine fibroid. As shown in Fig. 1, mRNA expression of IP3R1 and RYR1 was significant higher in fibroid than that of adjacent smooth muscle ($P < 0.05$). IP3R1