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EXPRESSION OF XANTHINE OXIDASE IN TESTICULAR CELLS

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Abstract

Objective : Previous studies showed that xanthine oxidase–related active oxygen generation was involved in heat stress–induced apoptosis in testicular cells. Hence, in the present study, the expression of xanthine oxidase in experimental cryptorchidism and heat–stressed testicular cells was assessed to determine the involvement of xanthine oxidase–related active oxygen generation in heat stress–induced apoptosis in testicular cells. **Methods :** (1) Immunohistological examination of xanthine oxidase was performed in a rat model of cryptorchidism. (2) mRNA expression of xanthine oxidase in cultured rat testicular cells was assessed by RT–PCR. (3) Expression of xanthine oxidase protein in cultured rat testicular cells was assessed by western blot analysis. **Results :** The presence of xanthine oxidase in testicular cells was confirmed by immunohistochemistry, especially after the spermatid stage. mRNA expression of xanthine oxidase in cultured testicular cells was similar with and without heat stress as determined by semiquantitative RT–PCR. Expression of xanthine oxidase protein in cultured testicular cells was similar under both conditions, i.e., at 32.5°C and at 37.0°C, as determined by western blot analysis. **Conclusions :** mRNA and protein expression of xanthine oxidase was confirmed in testicular cells. Quantitative changes in xanthine oxidase expression cause by heat stress have not been clarified. These findings suggest that activate oxygen generated by xanthine oxidase is involved in apoptosis of testicular cells induced by heat stress.

Key words : xanthine oxidase, male infertility, cryptorchidism, xanthine dehydrogenase, testis

Introduction

Spermatogenesis requires a temperature that is lower than body temperature. For that reason, the testes are located outside the abdominal cavity, i.e., in the scrotum. In fact, spermatogenesis is less efficient in cryptorchidism and in testes in temperatures comparable to that in the abdominal cavity[1-3].

Mammalian aerobic cells require oxygen, and reactive oxygen species (ROS) is generated when aerobic cells use oxygen for aerobic metabolism. ROS is harmful to vital cells, and it has been shown to induce apoptosis in cultured rat cells[4,5]. Many studies have suggested that when testicular cells are exposed to heat stress, an increase in active oxygen induces apoptosis in cultured testicular cells. Ikeda *et al.* demonstrated that heat stress induces apoptosis in cultured rat testicular cells, that apoptosis is suppressed by superoxide dismutase, and that oxidative stress alone induces apoptosis, thus revealing that an increase in ROS due to higher testicular temperatures is involved in heat stress–induced apoptosis in

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testicular cells[6]. Furthermore, Kumagai *et al.* showed that allopurinol, a xanthine oxidase inhibitor, suppresses heat stress induced apoptosis of testicular cells[7]. It has also been confirmed that BOF-4272, a specific inhibitor of xanthine oxidase, is important in suppressing heat stress-induced apoptosis in testicular cells[7]. Those findings suggest a close relationship between xanthine oxidase-related active oxygen generation and heat stress-induced apoptosis in the testes.

There have been few studies on xanthine oxidase in the testes. When a dose of cadmium sufficient to induce cancer in Leydig cells is administered, xanthine oxidase activity increases, but when insufficient cadmium is administered, xanthine oxidase activity does not increase. Several studies have suggested that carcinogenesis involves an increase in active oxygen-related enzymes, such as xanthine oxidase[8].

Many previous data have suggested the presence of xanthine oxidase activity in the testes of humans and other animals[9,10]. Based on these findings, we investigated mRNA and protein expression of xanthine oxidase in cultured testicular cells subjected to heat stress.

Methods

Culture medium and antibodies

F12-L15 medium (1 : 1 mixture of Ham's F12 and Lebovitz's L15 medium) and fetal bovine serum (FBS) used for testicular cell culture were purchased from Lifeteck Oriental Co. (Tokyo, Japan). Primary antibody (anti-rat xanthine oxidase) was kindly provided by Dr. Moriwaki (Histochem Cell Biol.)

Unilateral experimental cryptorchidism

Immature male Wistar rats (age, 40 days) were purchased from Clea Japan, Inc. (Tokyo, Japan). Unilateral cryptorchidism was experimentally induced in the rats under pentobarbital anesthesia according to previously described methods[11]. Briefly, a midline abdominal incision was made, and the left testis was displaced from the scrotum to the abdomen. After the gubernaculum was divide to prevent the testis from descending, the testis was fixed to the abdominal wall by suturing. The right testis remained in the scrotum as a euthermic control. The protocol for animal experimentation was approved by the Animal Research Center Committee of the Akita University School of Medicine.

Culture of testicular germ cells

Rats were killed by ether anesthesia, and testicular cells were isolated using a published method[12] with slight modifications. Briefly, testes were removed and decapsulated mechanically. Seminiferous tubules were gently excised and incubated in phosphate buffered saline (PBS) containing 0.25% collagenase (Type 1) for 8 min at 32.5°C with shaking. Then, the seminiferous tubules were washed, and again incubated in PBS containing 0.25% trypsin (Gibco, N.Y., USA) for 5 min at 32.5°C with shaking. After incubation, trypsin treatment was terminated by adding FBS at a final concentration of 10% (vol/vol). The resulting cell suspension was filtered through a 106-nm nylon mesh to remove cell aggregates and tissue debris, and then the cells were collected by centrifugation. Recovered cells were resuspended in F12-L15 medium supplemented with 1 mg/ml sodium bicarbonate, 100 IU/ml penicillin-G, 100 mg/ml of streptomycin sulphate, 14 ng/ml phenol red, and 10% FBS. The final concentration of testicular cells in the medium was adjusted to 5×10^6/ml. Two milliliters of cell suspension was plated in each well of 6-well C-1 plates (Sumitomo Verkleit Co., Tokyo, Japan), and cells were incubated in a humidified atmosphere of 5% CO_2 in air at 32.5°C, the optimal temperature for testicular germ cells, or 37.0°C.

RT-PCR

Total RNA was extracted from rat testes using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The quality of RNA in the extracts was assayed and the concentration was determined using a SmartSpec spectrophotometer (Bio-Rad Laboratories, Hercules, CA). RNA samples were reverse transcribed into cDNA and PCR proceeded as described in the legend to Table I. Rat liver cDNA served as a positive control, while water was substituted for mRNA in the negative control. PCR products were separated by 2% agarose gel electrophoresis (Agarose-LE, Nacalai Tesque, Inc., Kyoto, Japan) in the presence of ethidium bromide (Sigma) and were visualized using an ultra-violet

Table 1. Primers used for RT-PCR, PCR cycles, and temperatures for amplification for various cDNAs

Transcript	Primer sequence (5´-3´)	Product size (bp)
Xanthine oxidase	Sense : GCTTGAATCCTGCCATTGAT	401
	Antisense : AGTGACACACAGGGTGGTGA	
GAPDH	Sense : CATCACGCCACATTTCCC	182
	Antisense : GCAAGGTCATCCCTGAGCTGA	

PCR cycles : denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 60 s ; a total of 40 cycles was performed.

transilluminator (Funakoshi, Tokyo, Japan).

Immnohistochemistry

Rat testes were removed, had both ends cut and fixed in 20% formalin solution, were embedded in paraffin, and were then cut into 3-μm sections. These were collected on microslide glasses. Sections were deparffinized in xylene, rehydrated in a graded ethanol series, quenched in 3% hydrogen peroxidase, blocked and incubated with primary antibody (anti-rat xanthine oxidase) (kindly provided by Dr. Moriwaki, Histochem Cell Biol) overnight at 4°C. After several PBS washes, sections were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (DACO, Carpinteria, CA) for 1 h at room temperature, followed by 3,3´-diaminobenzidine, tetrahydrochloride, and were counterstained with haematoxylin. For controls, primary antibody was not used, and only peroxidase-conjugated goat anti-rabbit immunoglobulin G was applied.

Western blotting to detect xanthine oxidase protein

Protein extracted from testicular germ cells was subjected to Western blot analysis, as described previously[13]. Proteins were separated by SDS/PAGE using 6% polyacrylamide gels (Tefco, Tokyo, Japan). Resolved proteins were transferred to a polyvinylidene difluoride membrane using a Tefco electroblot apparatus and were then incubated with anti-rat xanthine oxidase antibody overnight at 4°C and with horse-radish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody for 1 h at room temperature. Immunoreactive bands were detected using Enhanced Chemuluminescence Plus Western blotting detection reagents (Amersham Biosciences, NJ) .

Results

Immunohistological examination of xanthine oxidase in experimental cryptorchidism model

Immunohistological examination showed that xanthine oxidase was expressed weakly in spermatogonia, spermatogenic cells, and spermatocytes in both descended and undescended testes, and expressed highly in testicular cells after the spermatid stage and in Leydig cells (Fig. 1). Beginning at three days after testicular retention surgery, apoptosis and cellular necrosis were seen, and xanthine oxidase was still expressed in the remaining spermatogonia and spermatogenic cells.

mRNA expression of xanthine oxidase in testicular cells with or without heat stress

To verify mRNA expression of xanthine oxidase in the testes, expression of mRNA extracted from testicular cells cultured for set periods with or without heat stress was investigated by RT-PCR. A 401-bp band indicating mRNA of xanthine oxidase was detected in mRNA samples extracted from rat liver as a positive control. The same bands were detected in each sample extracted from testicular cells cultured for set periods under two temperatures, i.e., at 32.5°C and at 37.0°C. RT-PCR was performed in order to semiquantitatively assess mRNA expression, but no quantitative differences were observed between the two temperatures. The same bands were detected in all samples, including GAPDH (Fig. 2).

Protein expression of xanthine oxidase in heat-stressed cultured testicular cells

To investigate the effect of heat stress on the level of xanthine oxidase, isolated testicular cells were incubated

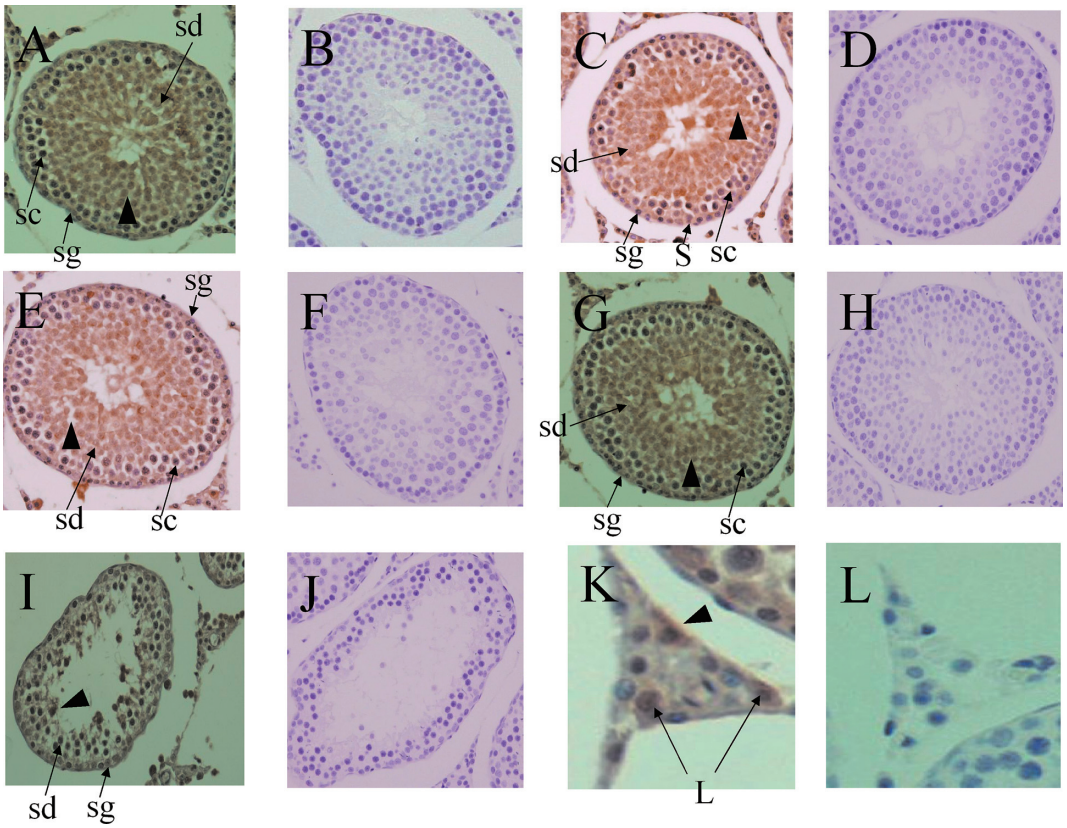

Fig. 1. Immunohistological examination of xanthine oxidase in experimental cryptorchidism model.
Immunostainning the testes in the scrotum and cryptorchidism using anti xanthine oxidase anibody. Xanthine oxidase weakly expressed in spermatogonia, spermatogenic cells and spermatocytes, but highly expressed in testicular cells after the spermatid stage. Xanthine oxidase also expressed in interstitial cells. ; A and B : Testis in scrotum C and D : Testis in scrotum for a day after operation, E and F : testis of cryptorchidism for a day after operation, G and H : Testis in scrotum for 3 days after operation, I and J : testis of cryptorchidism for 3 days after operation, K and L : Interstitial cells of testis in scrotum, ; A, C, E, G, I and K : immunostaining, B, D, F, H, J and L : control : S : Sertoli cell, sg : spermatogonia, sd : spermatid, sc : spermatocyte , L : Leydig cell ; ▲ : Closed arrows indicated positive cells. ; A–J magnification ×200, K and L : magnification ×400.

at either 32.5℃ or 37.0℃ for 12 hours and evaluated by western blot analysis. A 150-kDa band indicating protein expression of xanthine oxidase was detected in testicular cells before incubation. The same bands were detected in testicular cells cultured with or without heat stress for 12 hours (Fig. 3). Although 20 μg of protein from each sample were loaded in each lane, the band intensity of xanthine oxidase did not change under either condition.

Discussion

In the present study, expression of xanthine oxidase was confirmed in testicular cells after the spermatid stage and in Leydig cells. In addition, testicular cells death by apoptosis and expression of xanthine oxidase were not seen in the remaining spermatogonia and spermatogenic cells after testicular retention surgery, suggesting that heat stress does not affect localization of xanthine oxidase in the testes.

mRNA expression of xanthine oxidase was detected in

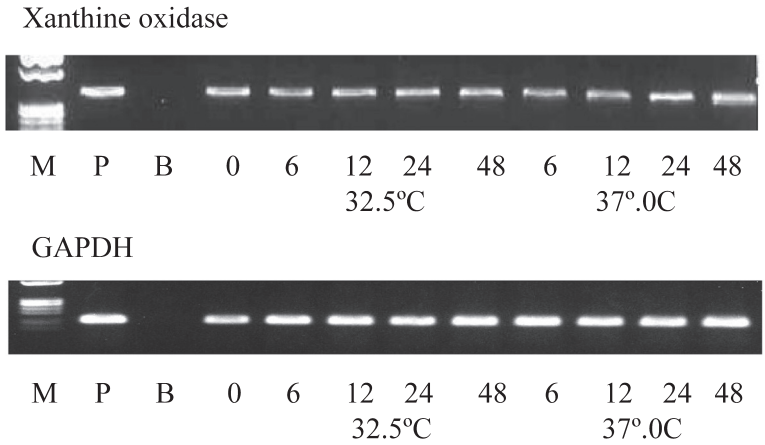

Fig. 2. mRNA expression of xanthine oxidase in cultured testicular cells with or without heat stress
A band indicate xanthine oxidase was seen at 401 bp. This band was seen in all samples, including GAPDH.
M : $\phi\chi$174–HaeIII digest ; P : the rats livers as positive control ; B : Blank without template

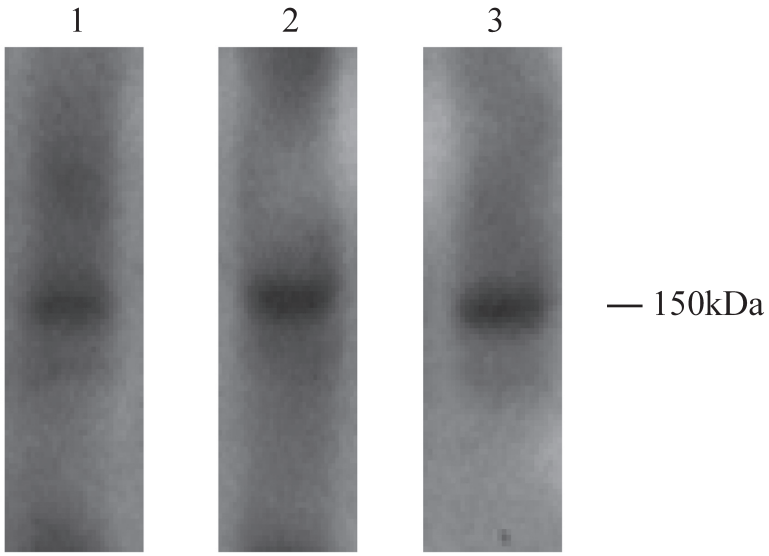

Fig. 3. Protein expression of xanthine oxidase as assessed by Western blotting using anti xanthine oxidase antibody. Each sample were extracted from cultured testicular cells before or after twelve hours incubation.
A band indicated xanthine oxidase was seen at 150 kDa. Each lane were loaded 20 μg of total protein.
Lane1 : before incubation, Lane2 : after incubation at 32.5°C, Lane3 : after incubation at 37.0°C for twelve hours.

cultured testicular cells before and after heat stress. Semiquantitative analysis did not show any marked differences in the mRNA expression of xanthine oxidase with respect to temperature. In western blotting examination, a 150–kDa band, indicating protein expression of xanthine oxidase, was detected in protein isolated from testicular cells before incubation and after incubation at 32.5°C or 37.0°C for 12 hours. These findings suggest that heat stress does not affect the protein expression of xanthine oxidase in the early stage of testicular cell death

cause by heat stress.

Xanthine oxidase is a highly versatile enzyme that is widely distributed among species and within various mammalian tissues. Xanthine oxidase is a dimer of approximately 150-kDa subunits, each containing one atom of molybdenum, one molecule of flavin adenine dinucleotide and two separate Fe2S2 clusters. It is most recognized for its role as the rate-limiting enzyme in nucleic acid degradation, through which all purines are channeled for terminal oxidation[14].

In the body, xanthine oxidase often exists as xanthine dehydrogenase[15]. In addition, xanthine dehydrogenase is converted to xanthine oxidase by modification of the protein molecule[16]. This conversion occurs either reversibly by oxidation of sulfhydryl residues or irreversibly by proteolysis[17]. Xanthine oxidase predominantly exists in vivo as an NAD^+-dependent dehydrogenase, and can be transformed into an oxygen-dependent oxidase by a variety of conditions ; for example, proteolysis, storage at −20°C, anaerobiosis, certain organic solvents, and sulfhydryl reagents. In addition, heat stress also causes this conversion.[18]

$$\text{hypoxanthine} + O_2 + H_2O \xrightarrow{XO} \text{xanthine} + H_2O_2 + O_2^-$$
$$\text{xanthine} + O2 + H_2O \xrightarrow{XO} \text{uric acid} + H_2O_2 + O_2^-$$

$$\text{hypoxanthine} + NAD^+ \xrightarrow{XDH} \text{xanthine} + NADH + H^+$$
$$\text{xanthine} + NAD^+ \xrightarrow{XDH} \text{uric acid} + NADH + H^+$$

The conversion of xanthine dehydrogenase to xanthine oxidase is accelerated by heat stress or subcellular incubation, and this suggests that the conversion of xanthine dehydrogenase to xanthine oxidase occurred in testes that were replaced in the abdominal cavity. Furthermore, xanthine oxidase activated active oxygen generation to increase active oxygen levels in the testes, and this facilitated testicular apoptosis. The same mechanism is at work in reperfusion injury of animal tissue, where a protease causes irreversible conversion of xanthine dehydrogenase to xanthine oxidase[19-23]. Furthermore, heat stress denatures adenine nucleotides to accumulate hypoxanthine. As large amounts of hypoxanthine are converted by xanthine oxidase, large amounts of active oxygen are produced during its conversion to xanthine and uric acid, thus inducing tissue damage[24]. In other words, two factors, i.e., conversion of xanthine dehydrogenase to xanthine oxidase and an increase in xanthine oxidase substrates, increase the activity of xanthine oxidase. In fact, when the levels of hypoxanthine, xanthine, and uric acid in cryptorchidism (days 1, 3, and 5) and normal testes were measured by high-performance liquid chromatography, the levels of these compounds in cryptorchidism were higher when compared to normal testes, and the degree of increase in uric acid was greater than that for hypoxanthine (data not shown).

These findings suggest that apoptosis induced by heat stress of testicular germ cell causes the conversion of xanthine oxidase. The activity of xanthine oxidase should be investigated according to different temperature levels.

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