Suppression of progesterone-enhanced hyperactivation in hamster spermatozoa by estrogen

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Abstract

In this study, I examined whether sperm hyperactivation in hamster is regulated by steroid hormones such as estrogen (estradiol, E_2) and progesterone. Although sperm hyperactivation was enhanced by progesterone, 17β -estradiol ($17\beta E_2$) itself did not affect sperm hyperactivation. However, $17\beta E_2$ suppressed progesterone-enhanced hyperactivation in a concentration-dependent manner through non-genomic pathways when spermatozoa were exposed to $17\beta E_2$ at the same time or before exposure to progesterone. When spermatozoa were exposed to $17\beta E_2$ after exposure to progesterone, $17\beta E_2$ did not suppress progesterone-enhanced hyperactivation. Moreover, 17α -estradiol, an inactive isomer of E_2 , did not suppress progesterone-enhanced hyperactivation. Observations using a FITC-conjugated $17\beta E_2$ showed that it binds to the acrosome region of the sperm head. Binding of $17\beta E_2$ to spermatozoa was not inhibited by progesterone, although $17\beta E_2$ did not suppress progesterone-enhanced hyperactivation when spermatozoa were exposed to $17\beta E_2$ after exposure to progesterone. On the other hand, binding of progesterone to spermatozoa was also not inhibited by $17\beta E_2$ even if progesterone-enhanced hyperactivation was suppressed by $17\beta E_2$. Although tyrosine phosphorylations of sperm proteins were enhanced by progesterone, enhancement of tyrosine phosphorylations by progesterone was suppressed by $17\beta E_2$. Moreover, tyrosine phosphorylations were inhibited by $17\beta E_2$ when only $17\beta E_2$ was added to the medium. From these results, it is likely that $17\beta E_2$ competitively suppresses progesterone-enhanced hyperactivation through the inhibition of tyrosine phosphorylations via non-genomic pathways.

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Introduction

After capacitation, mammalian spermatozoa fertilize eggs. Capacitated spermatozoa exhibit the acrosome reaction (AR) and hyperactivation. The AR is a modified exocytotic event involving the acrosome and the overlying sperm plasma membrane (Yudin *et al.* 1988, Yanagimachi 1994). The AR is required for penetration of the zona pellucida (ZP) of the egg and for sperm–egg plasma membrane fusion. Hyperactivation is a specialized movement of the sperm flagellum creating a propulsive force for passing through the ZP. Hyperactivated spermatozoa exhibit a large bend amplitude, whiplash and frenzied flagellar movements (Yanagimachi 1994, Fujinoki *et al.* 2001a, Suarez & Ho 2003).

Albumin, calcium and bicarbonate are important molecules in the capacitation process. Albumin promotes capacitation by removing cholesterol from the sperm plasma membrane (Langlais & Roberts 1985). Calcium and bicarbonate are involved in several intracellular signals such as the stimulation of adenylate cyclase, production of cAMP, and protein phosphorylation (Okamura *et al.* 1985, Visconti & Kopf 1998,

Visconti *et al.* 1998, Ho & Suarez 2001, Ho *et al.* 2002). For protein phosphorylations, it has been suggested that tyrosine phosphorylations are closely associated with capacitation (Visconti *et al.* 1995, Fujinoki *et al.* 2001b, 2006, Jha & Shivaji 2001). Moreover, 80 and/or 85 kDa tyrosine phosphorylations are regulated by calcium/calmodulin-dependent signals (Carrera *et al.* 1996) and protein phosphatase 1 (Suzuki *et al.* 2010), and are identified as A-kinase anchoring protein (AKAP; Carrera *et al.* 1996, Jha & Shivaji 2002).

Progesterone isolated from follicular fluid is a stimulator of human sperm AR (Osman *et al.* 1989). Progesterone stimulates the AR via non-genomic regulation (Baldi *et al.* 1998, 2009, Lösel & Wehling 2003, Luconi *et al.* 2004). In human spermatozoa, progesterone stimulates calcium influx, tyrosine phosphorylation, chloride efflux, and cAMP increase (Lösel & Wehling 2003, Harper *et al.* 2004, Luconi *et al.* 2004, Baldi *et al.* 2009). It also stimulates ZP penetration and the AR in hamster spermatozoa (Libersky & Boatman 1995b, Llanos & Anabalon 1996). Although spermatozoa do not have a classic intracellular progesterone receptor (PGR), a PGR is present in the cell membrane

(Sabeur *et al.* 1996, Baldi *et al.* 1998, 2009, Lösel & Wehling 2003, Luconi *et al.* 2004, Jang & Yi 2005). Moreover, it has been suggested that progesterone binds to the acrosome region, and PGR is localized to the same region in human (Gadkar *et al.* 2002) and hamster spermatozoa (Noguchi *et al.* 2008). Phospholipase C (PLC; Fukami *et al.* 2003) and/or protein kinase A (Harrison *et al.* 2000) are involved in progesterone-induced AR. Progesterone also changes motility parameters (Yang *et al.* 1994) and enhances hyperactivation in human (Sueldo *et al.* 1993) and hamster spermatozoa (Noguchi *et al.* 2008). Although progesterone enhances hyperactivation of human and hamster spermatozoa (Sueldo *et al.* 1993, Noguchi *et al.* 2008), the effective concentration of progesterone is different between humans and hamsters. For human spermatozoa, the effective concentration of progesterone for the enhancement of hyperactivation was several micrograms per milliliter, the same as the effective concentration for inducing the AR (Osman *et al.* 1989, Sueldo *et al.* 1993). On the other hand, for hamster spermatozoa, the effective concentration of progesterone for the enhancement of hyperactivation was 20 ng/ml (Noguchi *et al.* 2008). Moreover, at a concentration of 20 ng/ml, progesterone increased hamster sperm penetration and did not induce hamster sperm AR (Libersky & Boatman 1995b). At a concentration of 20 ng/ml, progesterone also enhanced and increased tyrosine phosphorylations associated with hyperactivation of hamster spermatozoa (Noguchi *et al.* 2008). The effective progesterone concentration for hamster sperm AR was also several micrograms per milliliter (Libersky & Boatman 1995b). Libersky & Boatman (1995a) measured progesterone concentration in fluids of reproductive organs, reporting that the progesterone concentration in hamster serum ranged from 5.56 to 12.85 ng/ml, the follicular fluid concentration ranged from 4.2 to 7.4 µg/ml, and the oviductal fluid concentration ranged from 44.04 to 175.06 ng/ml.

It has been suggested that 17β-estradiol (17βE₂) is also associated with a regulation of the AR (Luconi *et al.* 1999, 2004, Baldi *et al.* 2000, 2009). In human spermatozoa, 17βE₂ stimulates calcium influx and suppresses progesterone-induced AR (Luconi *et al.* 1999, Baldi *et al.* 2000). Estrogen receptors (ESRs) are also present in the cell membrane (Luconi *et al.* 1999, 2004, Baldi *et al.* 2000, 2009). Moreover, the suppression of progesterone-induced AR by 17βE₂ is non-genomic (Luconi *et al.* 2004, Baldi *et al.* 2009).

Although the AR is regulated by estrogen (E₂) and progesterone via non-genomic regulation, it is not known whether progesterone-enhanced hyperactivation is suppressed by 17βE₂. In the present study, I have shown that progesterone-enhanced hyperactivation and tyrosine phosphorylations are suppressed by 17βE₂ via non-genomic regulation.

Results

Suppression of progesterone-enhanced hyperactivation by 17βE₂

An earlier study suggested that hamster sperm hyperactivation was enhanced by 20 ng/ml progesterone via non-genomic regulation (Noguchi *et al.* 2008). It has been suggested that the AR is induced by progesterone and suppressed by 17βE₂ (Luconi *et al.* 1999, 2004, Baldi *et al.* 2000, 2009), so I examined whether hyperactivation was also enhanced by progesterone and suppressed by 17βE₂ using hamster spermatozoa.

Although 17βE₂ itself did not affect the percentage of motile spermatozoa and hyperactivation in hamster spermatozoa at all (Fig. 1), it affected progesterone-enhanced hyperactivation in a concentration-dependent manner (Figs 2 and 3). As shown in Fig. 2A and B, 17βE₂ suppressed progesterone-enhanced hyperactivation in a concentration-dependent manner, although it did not affect the percentage of motile spermatozoa when spermatozoa were exposed to 20 ng/ml progesterone after exposure to 20 ng/ml down to 2 fg/ml 17βE₂. When incubated for 1 (Fig. 2C), 1.5 (Fig. 2D), and 2 h (Fig. 2E), progesterone-enhanced hyperactivation was significantly suppressed by 20 ng/ml, 2 ng/ml, and 200 pg/ml 17βE₂ respectively. On the other hand, no suppression of progesterone-enhanced hyperactivation was observed at a concentration from 2 pg/ml down

Figure 1 Effects of 17βE₂ on the percentage of motile spermatozoa and hyperactivation. The percentage of motile spermatozoa (A) and hyperactivation (B) are shown when 17βE₂ was added to mTALP medium. Data are expressed as mean ± s.d. (Vehicle), mTALP + 0.1% (v/v) EtOH; (20 ng/ml E), mTALP + 20 ng/ml 17βE₂ + 0.1% (v/v) EtOH; (2 ng/ml E), mTALP + 2 ng/ml 17βE₂ + 0.1% (v/v) EtOH; (200 pg/ml E), mTALP + 200 pg/ml 17βE₂ + 0.1% (v/v) EtOH; (20 pg/ml E), mTALP + 20 pg/ml 17βE₂ + 0.1% (v/v) EtOH; (2 pg/ml E), mTALP + 2 pg/ml 17βE₂ + 0.1% (v/v) EtOH; (200 fg/ml E), mTALP + 200 fg/ml 17βE₂ + 0.1% (v/v) EtOH; (20 fg/ml E), mTALP + 20 fg/ml 17βE₂ + 0.1% (v/v) EtOH; (2 fg/ml E), mTALP + 2 fg/ml 17βE₂ + 0.1% (v/v) EtOH.

Figure 2 Effects of $17\beta E_2$ on the percentage of motile spermatozoa and progesterone-enhanced hyperactivation when spermatozoa were exposed to 20 ng/ml progesterone after exposure to $17\beta E_2$ for 5 min. The percentage of motile spermatozoa is shown as an overview of effects (A) when $17\beta E_2$ and progesterone were added to mTALP medium. The percentage of hyperactivation is shown as an overview of effects (B) and after incubation for 1 (C), 1.5 (D), and 2 (E) h after progesterone was added to the mTALP medium. Data are expressed as mean $\pm$ s.d. (Vehicle), mTALP + 0.2% (v/v) EtOH; (20 ng/ml progesterone), mTALP + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (20 ng/ml E), mTALP + 20 ng/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (2 ng/ml E), mTALP + 2 ng/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (200 pg/ml E), mTALP + 200 pg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (20 pg/ml E), mTALP + 20 pg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (2 pg/ml E), mTALP + 2 pg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (200 fg/ml E), mTALP + 200 fg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (2 fg/ml E), mTALP + 2 fg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH. *Significant difference compared with vehicle ($P < 0.05$); ^bSignificant difference compared with 20 ng/ml progesterone ($P < 0.05$); ^cSignificant difference compared with 20 ng/ml E ($P < 0.05$); ^dSignificant difference compared with 2 ng/ml E ($P < 0.05$). ^eSignificant difference compared with 200 pg/ml E ($P < 0.05$).

to 2 fg/ml $17\beta E_2$. Interestingly, the suppression of progesterone-enhanced hyperactivation by 20 pg/ml $17\beta E_2$ was midway between progesterone-enhanced hyperactivation and vehicle alone, with no significant difference to either. When spermatozoa were exposed to 20 ng/ml to 2 fg/ml $17\beta E_2$ and 20 ng/ml progesterone at the same time, $17\beta E_2$ suppressed progesterone-enhanced hyperactivation in a concentration-dependent manner, although it did not affect the percentage of motile spermatozoa (Fig. 3A and B). When incubated for 1 h (Fig. 3C), 1.5 h (Fig. 3D), and 2 h (Fig. 3E), 20 and 2 ng/ml $17\beta E_2$ significantly suppressed progesterone-enhanced hyperactivation. In contrast, 2 pg/ml to 1 fg/ml $17\beta E_2$ did not suppress progesterone-enhanced hyperactivation. When incubated for 1 h (Fig. 3C), the suppression of progesterone-enhanced hyperactivation by 200 and 20 pg/ml $17\beta E_2$ was midway between progesterone-enhanced hyperactivation and vehicle alone, with no significant difference to either. When incubated for 1.5 h

(Fig. 3D), the suppression of progesterone-enhanced hyperactivation by 200 pg/ml $17\beta E_2$ was midway between progesterone-enhanced hyperactivation and vehicle alone, with a significant difference to both. However, 20 pg/ml $17\beta E_2$ did not suppress progesterone-enhanced hyperactivation. When incubated for 2 h (Fig. 3E), 200 pg/ml $17\beta E_2$ significantly suppressed progesterone-enhanced hyperactivation, but 20 pg/ml $17\beta E_2$ had no effect. When spermatozoa were exposed to 20 ng/ml to 2 fg/ml $17\beta E_2$ after exposure to 20 ng/ml progesterone, $17\beta E_2$ did not affect the percentage of motile spermatozoa or progesterone-enhanced hyperactivation at all (Fig. 4).

The two isomers of E_2 are 17α -estradiol ($17\alpha E_2$) and $17\beta E_2$, of which $17\beta E_2$ alone has biological activity. When spermatozoa were exposed to 20 ng/ml progesterone after exposure to 20 ng/ml to 200 pg/ml $17\alpha E_2$, $17\alpha E_2$ had no effect on the percentage of motile spermatozoa or progesterone-enhanced hyperactivation

Figure 3 Effects of $17\beta E_2$ on the percentage of motile spermatozoa and progesterone-enhanced hyperactivation when spermatozoa were exposed to $17\beta E_2$ and 20 ng/ml progesterone at the same time. The percentage of motile spermatozoa is shown as an overview of effects (A) when $17\beta E_2$ and progesterone were added to mTALP medium. The percentage of hyperactivation is shown as an overview of effects (B) and after incubation for 1 (C), 1.5 (D), and 2 (E) h after progesterone was added to the mTALP medium. Data are expressed as mean $\pm$ s.d. (Vehicle), mTALP + 0.2% (v/v) EtOH; (20 ng/ml progesterone), mTALP + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (20 ng/ml E), mTALP + 20 ng/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (2 ng/ml E), mTALP + 2 ng/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (200 pg/ml E), mTALP + 200 pg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (20 pg/ml E), mTALP + 20 pg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (2 pg/ml E), mTALP + 2 pg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (200 fg/ml E), mTALP + 200 fg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (20 fg/ml E), mTALP + 20 fg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (2 fg/ml E), mTALP + 2 fg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH. ^aSignificant difference compared with vehicle ($P < 0.05$); ^bSignificant difference compared with 20 ng/ml progesterone ($P < 0.05$); ^cSignificant difference compared with 20 ng/ml E ($P < 0.05$); ^dSignificant difference compared with 2 ng/ml E ($P < 0.05$); ^eSignificant difference compared with 200 pg/ml E ($P < 0.05$).

(Fig. 5), whereas 20 ng/ml to 200 pg/ml $17\beta E_2$ suppressed progesterone-enhanced hyperactivation (Fig. 2B).

In general, $17\beta E_2$ affects cell functions via ESRs. Tamoxifen is an inhibitor of ESRs and was added to the modified Tyrode's albumin lactate pyruvate (mTALP) medium before $17\beta E_2$ and/or progesterone were added to mTALP medium. When spermatozoa were exposed to 1 μ mol/l tamoxifen before exposure to 20 ng/ml progesterone, tamoxifen had no effect on the percentage of motile spermatozoa, hyperactivation, or progesterone-enhanced hyperactivation (Fig. 6A and B). As shown in Figs 2 and 6D, $17\beta E_2$ suppressed progesterone-enhanced hyperactivation when spermatozoa were exposed to 20 ng/ml $17\beta E_2$ before exposure to 20 ng/ml progesterone. However, the suppression of progesterone-enhanced hyperactivation by $17\beta E_2$ was significantly inhibited by tamoxifen when spermatozoa were exposed to 1 μ mol/l tamoxifen before exposure to 20 ng/ml $17\beta E_2$ and 20 ng/ml progesterone (Fig. 6D). Under the same condition (Fig. 6C), tamoxifen had no effect on the percentage of motile spermatozoa.

Suppression of progesterone-enhanced hyperactivation by $17\beta E_2$ via non-genomic regulation

Sperm hyperactivation is enhanced by progesterone via non-genomic regulation associated with PGR (Noguchi *et al.* 2008). In the next step, I used FITC/BSA- $17\beta E_2$ to determine whether hamster sperm hyperactivation was regulated by $17\beta E_2$ via non-genomic regulation. Generally, $17\beta E_2$ enters the cell, binds to the intracellular ESRs, and induces gene expression (Baldi *et al.* 1998, 2009, Lösel & Wehling 2003, Luconi *et al.* 2004). However, FITC/BSA- $17\beta E_2$ cannot enter the cell because BSA blocks the entry of $17\beta E_2$ into the cell. It follows that the effects of FITC/BSA- $17\beta E_2$ occur through non-genomic signals.

As shown in Fig. 7A and B, 7.4 nmol/l, 740 pmol/l, and 74 pmol/l FITC/BSA- $17\beta E_2$, which are converted into $\sim$ 20 ng/ml, 2 ng/ml, and 200 pg/ml $17\beta E_2$ respectively did not affect the percentage of motile spermatozoa and hyperactivation, as with $17\beta E_2$ (Fig. 1). When spermatozoa were exposed to 20 ng/ml progesterone after exposure to 7.4 nmol/l, 740 pmol/l, and 74 pmol/l

Figure 4 Effects of $17\beta E_2$ on the percentage of motile spermatozoa and progesterone-enhanced hyperactivation when spermatozoa were exposed to $17\beta E_2$ after exposure to 20 ng/ml progesterone for 5 min. The percentage of motile spermatozoa (A) and hyperactivation (B) are shown when $17\beta E_2$ and progesterone were added to mTALP medium. Data are expressed as mean $\pm$ s.d. (Vehicle), mTALP + 0.2% (v/v) EtOH; (20 ng/ml progesterone), mTALP + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (20 ng/ml E), mTALP + 20 ng/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (2 ng/ml E), mTALP + 2 ng/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (200 pg/ml E), mTALP + 200 pg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (20 pg/ml E), mTALP + 20 pg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (2 pg/ml E), mTALP + 2 pg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (200 fg/ml E), mTALP + 200 fg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (20 fg/ml E), mTALP + 20 fg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (2 fg/ml E), mTALP + 2 fg/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH. All spermatozoa exposed to progesterone showed significant enhancement of hyperactivation compared with vehicle ($P < 0.05$). progesterone-enhanced hyperactivation was unaffected by $17\beta E_2$.

FITC/BSA- $17\beta E_2$, progesterone-enhanced hyperactivation was significantly suppressed by 7.4 nmol/l, 740 pmol/l, and 74 pmol/l FITC/BSA- $17\beta E_2$, although they did not affect the percentage of motile spermatozoa (Fig. 7C and D). The suppression of progesterone-enhanced hyperactivation by 7.4 nmol/l FITC/BSA- $17\beta E_2$ was significantly inhibited by 1 μ mol/l tamoxifen, although tamoxifen did not affect the percentage of motile spermatozoa (Fig. 7E and F).

Binding of $17\beta E_2$ to hamster spermatozoa

Progesterone binds to the acrosome region of the sperm head (Gadkar *et al.* 2002, Noguchi *et al.* 2008; Fig. 8Ac and Ad), although fluorescence of the middle piece of the sperm flagellum appears to be autofluorescence (Noguchi *et al.* 2008; Fig. 8Aa and Ab). As shown in Fig. 8Ae–h, it was observed that 7.4 nmol/l

FITC/BSA- $17\beta E_2$ bound to the acrosome region of the sperm head. Interestingly, 1 μ mol/l tamoxifen did not inhibit the binding of FITC/BSA- $17\beta E_2$ to the sperm head (Fig. 8B), although it did inhibit the effects of $17\beta E_2$ and FITC/BSA- $17\beta E_2$ on progesterone-enhanced hyperactivation (Figs 6 and 7).

When spermatozoa were exposed to 20 ng/ml $17\beta E_2$ before exposure to 20 ng/ml progesterone, progesterone-enhanced hyperactivation was suppressed by $17\beta E_2$ (Fig. 2). However, binding of 7 nmol/l FITC/BSA-P, converted $\sim$ 20 ng/ml progesterone, to the hamster sperm head was not inhibited by 20 ng/ml $17\beta E_2$ when spermatozoa were exposed to 20 ng/ml $17\beta E_2$ before exposure to 7 nmol/l FITC/BSA-P (Fig. 8Ca–d). On the other hand, progesterone-enhanced hyperactivation was not suppressed by $17\beta E_2$ when spermatozoa were exposed to 20 ng/ml $17\beta E_2$ after exposure to 20 ng/ml progesterone (Fig. 4). Binding of 7.4 nmol/l FITC/BSA- $17\beta E_2$ to the sperm head was not suppressed by 20 ng/ml progesterone when spermatozoa were exposed to 7.4 nmol/l FITC/BSA- $17\beta E_2$ after exposure to 20 ng/ml progesterone (Fig. 8Ce–h).

Figure 5 Effects of $17\alpha E_2$ on the percentage of motile spermatozoa and progesterone-enhanced hyperactivation. After exposure to $17\alpha E_2$ for 5 min, spermatozoa were exposed to 20 ng/ml progesterone. The percentage of motile spermatozoa (A) and hyperactivation (B) are shown when $17\alpha E_2$ and progesterone were added to mTALP medium. Data are expressed as mean $\pm$ s.d. (Vehicle), mTALP + 0.2% (v/v) EtOH; (20 ng/ml progesterone), mTALP + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (20 ng/ml E), mTALP + 20 ng/ml $17\alpha E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (2 ng/ml E), mTALP + 2 ng/ml $17\alpha E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH; (200 pg/ml E), mTALP + 200 pg/ml $17\alpha E_2$ + 20 ng/ml progesterone + 0.2% (v/v) EtOH. All spermatozoa exposed to progesterone showed significant enhancement of hyperactivation compared with vehicle ($P < 0.05$). progesterone-enhanced hyperactivation was unaffected by $17\alpha E_2$.

Figure 6 Inhibition of suppression of progesterone-enhanced hyperactivation by $17\beta E_2$. After exposure to $1 \mu\text{mol/l}$ tamoxifen for 5 min, spermatozoa were exposed to 20 ng/ml $17\beta E_2$. After incubation for 5 min, they were exposed to 20 ng/ml progesterone. The percentage of motile spermatozoa (A) and hyperactivation (B) are shown when tamoxifen and progesterone were added to the mTALP medium. The percentage of motile spermatozoa (C) and hyperactivation (D) are shown when tamoxifen, $17\beta E_2$, and progesterone were added to mTALP medium. Data are expressed as mean $\pm$ s.d. (Vehicle), mTALP + 0.1% (v/v) ethanol + 0.1% (v/v) DMSO; (P), mTALP + 20 ng/ml progesterone + 0.1% (v/v) EtOH + 0.1% (v/v) DMSO; (T), mTALP + $1 \mu\text{mol/l}$ tamoxifen + 0.1% (v/v) EtOH + 0.1% (v/v) DMSO; (P + T), mTALP + $1 \mu\text{mol/l}$ tamoxifen + 20 ng/ml progesterone + 0.1% (v/v) EtOH + 0.1% (v/v) DMSO; (P + E), mTALP + 20 ng/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.1% (v/v) EtOH + 0.1% (v/v) DMSO; (P + E + T), mTALP + $1 \mu\text{mol/l}$ tamoxifen + 20 ng/ml $17\beta E_2$ + 20 ng/ml progesterone + 0.1% (v/v) EtOH + 0.1% (v/v) DMSO. ^aSignificant difference compared with vehicle ($P < 0.05$); ^bSignificant difference compared with T ($P < 0.05$); ^cSignificant difference compared with progesterone + E ($P < 0.05$).

Effects of $17\beta E_2$ on tyrosine phosphorylations

It is accepted that tyrosine phosphorylations are an important event in sperm hyperactivation (Yanagimachi 1994, Visconti *et al.* 1995, Visconti & Kopf 1998, Fujinoki *et al.* 2006, Fujinoki 2009). A previous study (Noguchi *et al.* 2008) suggested that tyrosine phosphorylations in hamster spermatozoa were enhanced by 20 ng/ml progesterone.

As shown in Fig. 9B, several tyrosine phosphorylations occurred during hyperactivation in a time-dependent manner. Four tyrosine phosphorylations, designated as 80u, 85u, 80ut, and 85ut (Fujinoki *et al.* 2006), were enhanced by 20 ng/ml progesterone (Noguchi *et al.* 2008; Fig. 9D). Phosphorylations of 80u and 85u were detected in the urea extract (Fujinoki *et al.* 2006; Fig. 9B lanes a–f). Phosphorylations of 80ut and 85ut were detected in the urea–thiourea extract (Fujinoki *et al.* 2006; Fig. 9B lanes g–l).

When hamster spermatozoa were exposed to 20 ng/ml $17\beta E_2$, tyrosine phosphorylations including 80u and 85u were not detected in the urea extract (Fig. 9C lanes a–f), although 20 ng/ml $17\beta E_2$ itself did not affect the percentage of motile spermatozoa and hyperactivation (Fig. 1). On the other hand, tyrosine phosphorylations were detected in the urea–thiourea extract when spermatozoa were exposed to 20 ng/ml $17\beta E_2$ (Fig. 9C lanes g–l). When spermatozoa were exposed to 20 ng/ml progesterone, tyrosine phosphorylations of 80u, 85u, 80ut and 85ut were enhanced when compared with vehicle (Fig. 9B and D). As shown in

Fig. 9E lanes a–f, tyrosine phosphorylations were not detected in the urea extract when spermatozoa were exposed to 20 ng/ml $17\beta E_2$ before they were exposed to 20 ng/ml progesterone. The tyrosine phosphorylations detected in the urea–thiourea extract, as shown in Fig. 9E lanes g–l, were the same as the tyrosine phosphorylations as shown in Fig. 9C lanes g–l. Under the same conditions, progesterone-enhanced hyperactivation was suppressed (Fig. 2). As shown in Fig. 6, $1 \mu\text{mol/l}$ tamoxifen canceled the effect of 20 ng/ml $17\beta E_2$ on progesterone-enhanced hyperactivation. For tyrosine phosphorylations, $1 \mu\text{mol/l}$ tamoxifen inhibited the effect of 20 ng/ml $17\beta E_2$ (Fig. 9F).

Discussion

The AR is induced in a ligand-dependent manner. Progesterone is a major ligand of the AR (Osman *et al.* 1989, Baldi *et al.* 1998, 2009, Lösel & Wehling 2003, Luconi *et al.* 2004). On the other hand, progesterone also enhances sperm hyperactivation (Sueldo *et al.* 1993, Noguchi *et al.* 2008). Recently, melatonin was also shown to be a ligand that enhances sperm hyperactivation in hamster (Fujinoki 2008). It would therefore appear that hyperactivation is enhanced by ligands (Fujinoki 2009).

Progesterone-induced AR is suppressed by $17\beta E_2$ through non-genomic regulation in human spermatozoa (Luconi *et al.* 1999, 2004, Baldi *et al.* 2000, 2009). In the present study, I showed that $17\beta E_2$ suppresses progesterone-enhanced hyperactivation through

Figure 7 Suppression of progesterone-enhanced hyperactivation by $17\beta E_2$ via non-genomic regulation. After exposure to $1 \mu\text{mol/l}$ tamoxifen for 5 min, spermatozoa were exposed to FITC/BSA- $17\beta E_2$. After incubation for 5 min, they were exposed to 20 ng/ml progesterone. The percentage of motile spermatozoa (A) and hyperactivation (B) are shown when only FITC/BSA- $17\beta E_2$ was added to mTALP medium. The percentage of motile spermatozoa (C) and hyperactivation (D) are shown when FITC/BSA- $17\beta E_2$ and progesterone were added to mTALP medium. The percentage of motile spermatozoa (E) and hyperactivation (F) are shown when tamoxifen, FITC/BSA- $17\beta E_2$, and progesterone were added to mTALP medium. Data are expressed as mean $\pm$ s.d. (Vehicle), mTALP + 0.1% (v/v) ethanol + 0.1% (v/v) DMSO; (20 ng/ml progesterone or progesterone), mTALP + 20 ng/ml progesterone + 0.1% (v/v) EtOH + 0.1% (v/v) DMSO; (7.4 nmol/l BSA-E or progesterone + BSA-E), mTALP + 7.4 nmol/l FITC/BSA- $17\beta E_2$ + 20 ng/ml progesterone + 0.1% (v/v) EtOH + 0.1% (v/v) DMSO; (740 pmol/l BSA-E), mTALP + 740 pmol/l FITC/BSA- $17\beta E_2$ + 20 ng/ml progesterone + 0.1% (v/v) EtOH + 0.1% (v/v) DMSO; (74 pmol/l BSA-E), mTALP + 74 pmol/l FITC/BSA- $17\beta E_2$ + 20 ng/ml progesterone + 0.1% (v/v) EtOH + 0.1% (v/v) DMSO; (P + BSA-E + T), mTALP + $1 \mu\text{mol/l}$ tamoxifen + 7.4 nmol/l FITC/BSA- $17\beta E_2$ + 20 ng/ml progesterone + 0.1% (v/v) EtOH + 0.1% (v/v) DMSO. ^aSignificant difference compared with vehicle ($P < 0.05$); ^bSignificant difference compared with 7.4 nmol/l FITC/BSA-E or progesterone + FITC/BSA-E ($P < 0.05$); ^cSignificant difference compared with 740 pmol/l FITC/BSA-E ($P < 0.05$); ^dSignificant difference compared with 74 pmol/l FITC/BSA-E ($P < 0.05$).

non-genomic regulation in hamster spermatozoa (see Figs 2, 3, 6 and 7). Since $17\beta E_2$ binds to the acrosome region of the sperm head (see Fig. 8), it appears that signals of $17\beta E_2$ start at the sperm head and are transmitted to the sperm flagellum. Calcium is a key molecule in the regulation of AR and hyperactivation by steroid hormones (Luconi *et al.* 1999, 2004, Baldi *et al.* 2000, 2009, Noguchi *et al.* 2008, Fujinoki 2009). After progesterone binds to the sperm head, it stimulates calcium influx and activation of PLC (Fukami *et al.* 2003, Lösel & Wehling 2003, Harper *et al.* 2004, Luconi *et al.* 2004, Noguchi *et al.* 2008, Baldi *et al.* 2009, Fujinoki 2009). Moreover, spermatozoa are not hyperactivated in the mTALP medium without calcium (Fujinoki 2008, Noguchi *et al.* 2008). In human spermatozoa, $17\beta E_2$ also stimulates calcium influx and suppresses progesterone-induced AR (Luconi *et al.* 1999, 2004, Baldi *et al.* 2000, 2009). Although the $17\beta E_2$ signal is unknown, it is likely that the $17\beta E_2$ signal differs from progesterone signals and suppresses progesterone signals via calcium influx

(Luconi *et al.* 1999, 2004, Baldi *et al.* 2000, 2009). In the present study, I did not examine calcium influx induced by $17\beta E_2$ in hamster spermatozoa. Based on the assumption that $17\beta E_2$ stimulates calcium influx, in a future study, I will investigate the relationship between $17\beta E_2$ and calcium influx using hamster spermatozoa.

When spermatozoa are hyperactivated, a major intracellular signaling event that occurs is tyrosine phosphorylation (Yanagimachi 1994, Visconti & Kopf 1998, Visconti *et al.* 1998, Ho & Suarez 2001, Ho *et al.* 2002, Fujinoki *et al.* 2006, Fujinoki 2009). As part of progesterone-enhanced hyperactivation, progesterone enhances tyrosine phosphorylations of 80 and 85 kDa proteins such as 80u, 85u, 80ut, and 85ut (Noguchi *et al.* 2008; see Figs 2 and 9). Interestingly, $17\beta E_2$ partially suppressed tyrosine phosphorylations (see Fig. 9C), and $17\beta E_2$ suppressed progesterone-enhanced tyrosine phosphorylations (see Fig. 9E). Tamoxifen inhibited the suppression of progesterone-enhanced tyrosine phosphorylations by $17\beta E_2$ (see Fig. 9F), although it did not

Figure 8 Binding of steroid hormones to spermatozoa. (A) Binding of $17\beta E_2$ and progesterone to sperm head. (a and b) Hamster spermatozoa were incubated in mTALP medium with 0.1% (v/v) EtOH and 0.1% (v/v) DMSO. (c and d) Hamster spermatozoa were incubated in mTALP with 7 nmol/l FITC/BSA-P, 0.1% (v/v) EtOH, and 0.1% (v/v) DMSO. (e–h) Hamster spermatozoa were incubated in mTALP medium with 7.4 nmol/l FITC/BSA- $17\beta E_2$, 0.1% (v/v) EtOH, and 0.1% (v/v) DMSO. (B) Effect of tamoxifen on binding of $17\beta E_2$ to the sperm head. (a–d) Hamster spermatozoa were incubated in mTALP medium with 7.4 nmol/l FITC/BSA- $17\beta E_2$, 1 μ mol/l tamoxifen, 0.1% (v/v) EtOH, and 0.1% (v/v) DMSO. (e–h) Hamster spermatozoa were incubated in mTALP medium with 1 μ mol/l tamoxifen, 0.1% (v/v) EtOH, and 0.1% (v/v) DMSO. (C) Effect of steroid hormones on binding of steroid hormones to the sperm head. (a–d) Hamster spermatozoa were incubated in mTALP medium with 7 nmol/l FITC/BSA-P after exposure to 20 ng/ml $17\beta E_2$, 0.1% (v/v) EtOH, and 0.1% (v/v) DMSO. (e–h) Hamster spermatozoa were incubated in mTALP medium with 7.4 nmol/l FITC/BSA- $17\beta E_2$ after exposure to 20 ng/ml progesterone, 0.1% (v/v) EtOH, and 0.1% (v/v) DMSO. (a, c, e, g in A–C) Observed under visible light; (b, d, f, h in A–C) observed under fluorescence. Fluorescence of the mitochondrial sheath in the flagellum appeared to be autofluorescence. Bar represents 100 μ m.

inhibit the binding of $17\beta E_2$ at the sperm head (see Fig. 8B). Tyrosine phosphorylation of proteins detected in the urea extract was suppressed by $17\beta E_2$ (see Fig. 9C). The urea extract contained proteins from many sperm structures, except the fibrous sheath and head

(Fujinoki *et al.* 2006). On the other hand, tyrosine phosphorylation of proteins detected in the urea–thiourea extract was unaffected (see Fig. 9C). The urea–thiourea extract contained proteins of fibrous sheath (Fujinoki *et al.* 2006). One of the tyrosine phosphorylations

Figure 9 Changes in tyrosine phosphorylations associated with hyperactivation regulated by steroid hormones. Tyrosine phosphorylations were detected by western blotting using anti-phosphotyrosine MAB (clone, PT-66). (A) Typical CBB-stained membrane after blotting. (B) Western blotting against proteins obtained from spermatozoa that were incubated in mTALP medium with 0.2% (v/v) EtOH and 0.1% (v/v) DMSO. (C) Western blotting against proteins obtained from spermatozoa that were incubated in mTALP medium with 20 ng/ml $17\beta E_2$, 0.2% (v/v) EtOH, and 0.1% (v/v) DMSO. (D) Western blotting against proteins obtained from spermatozoa that were incubated in mTALP medium with 20 ng/ml progesterone, 0.2% (v/v) EtOH, and 0.1% (v/v) DMSO. (E) Western blotting against proteins obtained from spermatozoa that were incubated in mTALP medium with 20 ng/ml $17\beta E_2$, 20 ng/ml progesterone, 0.2% (v/v) EtOH, and 0.1% (v/v) DMSO. Hamster spermatozoa were exposed to 20 ng/ml progesterone after exposure to 20 ng/ml $17\beta E_2$ for 5 min. (F) Western blotting against proteins obtained from spermatozoa that were incubated in mTALP medium with 20 ng/ml $17\beta E_2$, 20 ng/ml progesterone, 1 $\mu\text{mol/l}$ tamoxifen, 0.2% (v/v) EtOH, and 0.1% (v/v) DMSO. Hamster spermatozoa were exposed to 20 ng/ml $17\beta E_2$ after exposure to 1 $\mu\text{mol/l}$ tamoxifen for 5 min. After incubation for 5 min, they were exposed to 20 ng/ml progesterone. Lanes a–f and g–l illustrate results obtained from the urea extract and the urea–thiourea extract respectively. Lanes a and g were incubated for 0 h, lanes b and h were incubated for 0.5 h, lanes c and i were incubated for 1 h, lanes d and j were incubated for 2 h, lanes e and k were incubated for 3 h, and lanes f and l were incubated for 4 h. SDS-PAGE was performed on 5 μl samples per lane. Arrows show tyrosine phosphorylation of 80 and 85 kDa proteins. Numbers on the left side of (A) indicate the molecular weight standard.

associated with hyperactivation is that of AKAP, 80 and/or 85 kDa components of the fibrous sheath (Carrera *et al.* 1996, Jha & Shivaji 2002). Therefore, tyrosine phosphorylations of 80 and/or 85 kDa proteins detected in the urea–thiourea extract (80ut and 85ut) corresponded to AKAP and were not affected by $17\beta E_2$, although they were affected by progesterone (Noguchi *et al.* 2008; see Fig. 9C and D). Because hamster spermatozoa were hyperactivated in the presence of $17\beta E_2$, it is likely that tyrosine phosphorylations of 80ut and 85ut are associated with the essential regulatory mechanisms of hyperactivation. Because tyrosine phosphorylation of AKAP is regulated by calcium signals associated with calcium/calmodulin-dependent protein kinase (Carrera *et al.* 1996), it seems that tyrosine phosphorylations of 80ut and 85ut are regulated by calcium signals associated with calcium/calmodulin-dependent kinase. At least, tyrosine phosphorylations of 80ut and 85ut were enhanced by progesterone through calcium signals associated with PLC (Noguchi *et al.* 2008). On the other hand, tyrosine phosphorylations of 80 and 85 kDa proteins detected in the urea extract (80u and 85u) were suppressed by $17\beta E_2$ and enhanced by progesterone (Noguchi *et al.* 2008; see Fig. 9C and D). Even if 80u, 85u, and other proteins detected in the urea extract were not phosphorylated at tyrosine residues by $17\beta E_2$, spermatozoa were hyperactivated (see Figs 2 and 9C). These results suggest that tyrosine phosphorylations

detected in the urea extract are not associated with the essential regulatory mechanisms of hyperactivation. Because tyrosine phosphorylations detected in the urea extract responded to the effects of steroid hormones, however, it is likely that they are associated with modulatory mechanisms of hyperactivation. Although tyrosine phosphorylations of 80u and 85u are enhanced by progesterone (Noguchi *et al.* 2008; see Fig. 9D), their regulatory mechanisms are not known at all and have not been identified yet. In a future study, I will investigate the regulatory mechanism of tyrosine phosphorylation of 80u and 85u and their identification.

In the present study, I demonstrated that enhancement of sperm hyperactivation was competitively regulated by steroid hormones (see Figs 2 and 3). It is suggested that the AR is also competitively regulated by steroid hormones (Luconi *et al.* 1999, 2004, Baldi *et al.* 2000, 2009). For both the AR and the hyperactivation, progesterone enhances the functions and $17\beta E_2$ suppresses the effects of progesterone (Luconi *et al.* 1999, 2004, Baldi *et al.* 2000, 2009, Noguchi *et al.* 2008, Fujinoki 2009; see Figs 2 and 3). The hyperactivation threshold concentration of $17\beta E_2$ was 20 pg/ml when spermatozoa were exposed to $17\beta E_2$ before exposure to 20 ng/ml progesterone (see Fig. 2). When spermatozoa were exposed to both steroid hormones at the same time, the threshold concentration of $17\beta E_2$ was 200 pg/ml (see Fig. 3). Female mammals have an estrous or menstrual

cycle, causing concentrations of $17\beta E_2$ and progesterone in the blood and tissues to fluctuate. The balance between the concentrations of $17\beta E_2$ and progesterone accordingly appears to regulate sperm AR and hyperactivation. When $17\beta E_2$ is more dominant over progesterone, enhancement of AR and hyperactivation is suppressed. Conversely, AR and hyperactivation are enhanced when progesterone is dominant over $17\beta E_2$. In a future study, I will examine the changes in sperm function under different patterns of $17\beta E_2$ and progesterone concentrations.

Materials and Methods

Chemicals

Anti-phosphotyrosine MAB (clone, PT-66), $17\alpha E_2$, $17\beta E_2$, progesterone, FITC-conjugated and BSA-conjugated $17\beta E_2$ (FITC/BSA- $17\beta E_2$), FITC-conjugated and BSA-conjugated progesterone (FITC/BSA-P), and tamoxifen were purchased from Sigma Chemical Company. BSA fraction V and Nonidet P-40 (NP-40) were purchased from Merck KGaA. ECL Plus kits and films for ECL were purchased from Amersham Biosciences. Other reagent grade chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animals and preparation of hyperactivated spermatozoa

Spermatozoa were obtained from the caudal epididymis of sexually mature (12–20 weeks old) male golden hamsters (*Mesocricetus auratus*). Hamsters were housed in accordance with the guidelines of the Dokkyo Medical University and the Laboratory Animal Research Center in Dokkyo Medical University of the care and use of laboratory animals.

Hyperactivated spermatozoa were prepared according to the method described previously (Fujinoki *et al.* 2006), using modified TALP (mTALP) medium containing 101.02 mmol/l NaCl, 2.68 mmol/l KCl, 2 mmol/l $CaCl_2$, 1.5 mmol/l $MgCl_2 \cdot 6H_2O$, 0.36 mmol/l $NaH_2PO_4 \cdot 2H_2O$, 35.70 mmol/l $NaHCO_3$, 4.5 mmol/l D-glucose, 0.09 mmol/l sodium pyruvate, 9 mmol/l sodium lactate, 0.5 mmol/l hypotaurine, 0.05 mmol/l (–)epinephrine, 0.2 mmol/l sodium taurocholic acid, 5.26 μ mol/l sodium metabisulfite, 0.05% (w/v) streptomycin sulfate, 0.05% (w/v) potassium penicillin G, and 15 mg/ml BSA (pH 7.4 at 37 °C under 5% (v/v) CO_2 in air). An aliquot of caudal epididymal spermatozoa was placed in the bottom of a test tube, and several milliliters of the mTALP medium were carefully added, followed by incubation for 5 min to allow spermatozoa to swim up. The supernatant containing motile spermatozoa was collected, placed on a culture plate (35 mm dish), and incubated for 4 h at 37 °C under 5% (v/v) CO_2 in air to achieve hyperactivation. Steroid hormones and tamoxifen were dissolved in ethanol (EtOH) and dimethyl sulfoxide (DMSO) respectively, and then added to the mTALP medium after placing the motile spermatozoa on the culture plate.

Measurement of the percentage of motile spermatozoa and hyperactivation

The percentage of motile spermatozoa and hyperactivation measurements were performed according to the method described in previous studies (Fujinoki *et al.* 2001b, 2006). Hamster spermatozoa suspended in the mTALP medium were diluted 10-fold and placed on the culture plate (35 mm dish). Motile and hyperactivated spermatozoa were recorded on a DVD using a CCD camera (Progressive 3CCD, Sony Corp., Tokyo, Japan) attached to a microscope (IX70, Olympus Corp., Tokyo, Japan) with phase-contrast illumination and a small CO_2 incubator (MI-IBC, Olympus). Each observation was performed at 37 °C and recorded for 2 min, and the analyses comprised counting the numbers of total spermatozoa, motile spermatozoa, and hyperactivated spermatozoa in 20 different fields. The percentage of motile spermatozoa and hyperactivation were respectively defined as (the number of motile spermatozoa/the number of total spermatozoa) $\times$ 100 and (the number of hyperactivated spermatozoa/numbers of total spermatozoa) $\times$ 100. Statistical analyses were performed using the *post hoc* test of ANOVA.

Ligand assays of $17\beta E_2$ and progesterone

Ligand assays of $17\beta E_2$ and progesterone were performed according to the method described previously (Noguchi *et al.* 2008). An aliquot of caudal epididymal spermatozoa was placed on the bottom of a test tube, and several milliliters of mTALP medium with FITC/BSA- $17\beta E_2$ or FITC/BSA-P were carefully added, followed by incubation for 5 min to allow spermatozoa to swim up. The supernatant containing motile spermatozoa was collected, placed on a culture dish, and incubated for 5 min at 37 °C under 5% (v/v) CO_2 in air. After incubation, several microliters of the supernatant were placed on a glass slide without fluorescence and observed using a light microscope (IX70, Olympus) with phase-contrast illumination and a fluorescence unit.

Preparation of sperm protein extracts

Sperm proteins were extracted according to the method described previously (Fujinoki *et al.* 2006). In brief, spermatozoa were suspended at 30 mg spermatozoa/ml in the urea solution containing 7 mol/l urea and 10% (v/v) 2-mercaptoethanol. After pipetting, the suspension was incubated on ice for 10 min. Following centrifugation at 15 000 *g* for 10 min at 4 °C, the supernatant was used as the urea extract. The precipitate was resuspended in the same volume of urea–thiourea solution containing 5 mol/l urea, 1 mol/l thiourea, 10% (v/v) 2-mercaptoethanol, and 2% (v/v) NP-40 as the urea solution. After pipetting, the suspension was incubated on ice for 10 min, centrifuged at 15 000 *g* for 10 min at 4 °C, and the supernatant was used as the urea–thiourea extract.

SDS-PAGE

SDS-PAGE was performed using the Laemmli protocol (Laemmli 1970) using a separating gel of 10% (w/v) polyacrylamide containing 0.1% (w/v) SDS.

Western blotting

Western blotting was performed according to the method described previously (Fujinoki *et al.* 2001b, 2006). The blotted membrane was blocked with 5% (w/v) skim milk in Tris-buffered saline (TBS) containing 0.15 mol/l NaCl and 20 mmol/l Tris-HCl (pH 7.5) for 1 h at 20 °C, and incubated with anti-phosphotyrosine MAB (clone, PT-66) (1:1000 dilution with 5% (w/v) skim milk in TBS) for 1 h at 20 °C. Following a TBS wash, the membrane was incubated with anti-mouse IgG antibody-conjugated peroxidase (1:5000 dilution with 5% (w/v) skim milk in TBS). Color reactions were conducted using the ECL Plus kit.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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