

Dynamic aspects of ovarian superoxide dismutase isozymes during the ovulatory process in the rat

Eisuke F. Sato^a, Hirotugu Kobuchi^b, Keisuke Edashige^a, Masahiko Takahashi^c, Tamotsu Yoshioka^b, Kozo Utsumi^a and Masayasu Inoue^d

Departments of ^aMedical Biology and ^cNeuropsychiatry, Kochi Medical School, Nankoku, Kochi 783, Japan, ^bCenter for Adult Diseases, Kurashiki, Kurashiki 710, Japan and ^dDepartment of Biochemistry, Kumamoto University Medical School, 2-2-1 Honjo, Kumamoto 860, Japan

Received 13 March 1992; revised version received 3 April 1992

To investigate the role of superoxide dismutase (SOD) in the ovulatory process, SOD isozymes and their mRNAs were determined in the ovary of 22-day-old rat. After treatment with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG), ovarian activity of Mn-SOD decreased markedly while Cu/Zn-SOD remained unchanged. However, the ovarian level of mRNA for Mn-SOD markedly increased after hCG-treatment while that for Cu/Zn-SOD decreased only slightly. Ovulation was inhibited by intravenous injection of a long-acting SOD. These results suggested that superoxide radicals in the ovary might play a critical role in the mechanism for hCG-induced ovulation.

Superoxide; Ovulation; Cu/Zn-SOD; Mn-SOD; mRNA; Rat ovary

1. INTRODUCTION

Reactive oxygen species, such as superoxide, have been postulated to underlie the pathogenesis of various diseases including inflammation [1,2]. In fact, Pederson and Aust [3] reported that superoxide induced lipid peroxidation of biological membranes by an SOD-inhibitable mechanism. Hence, superoxide dismutase (SOD) and related enzymes are believed to play critical roles in protecting cells from hazardous oxygen species. However, it should be noted that reactive oxygen species also play important roles in cellular defense mechanism including bactericidal action of leukocytes [4].

Ovulation occurs by a mechanism similar to that for inflammatory reactions which enhance the synthesis of prostaglandins [5], histamine [6], and bradykinin [7]. Furthermore, gonadotropin-induced ovulatory surge triggered an inflammatory reaction in mature follicles resulting in the rupture of their walls and that non-steroidal anti-inflammatory drugs inhibited the ovulation [8]. Laloraya et al. [9] demonstrated that ovarian levels of SOD and superoxide radicals changed inversely during the reproductive cycle of the rat. However, the role of superoxide radical and/or its meta-

bolites in the ovulatory process is not known. The present study reports changes in ovarian activity of SOD isozymes and expression of their mRNAs during the ovulatory process in the rat.

2. MATERIALS AND METHODS

2.1. Materials

Experiments for ovulation were carried out with 22-day-old immature female Wistar rats. Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from Teikoku-Zoki Co. Long-acting SOD (SM-SOD) was synthesized from human Cu/Zn-SOD and poly(styrene-co-maleic acid) butyl ester derivative (SM) as described [10]. Inactivation of SM-SOD was carried out by incubating the enzyme in 0.1 M H₂O₂ at 25°C for 6 h [11]. After extensive dialysis against saline solution, the H₂O₂-inactivated SM-SOD was used for experiments. Megaprime DNA labeling system was obtained from Amersham Japan (Tokyo). [α -³²P]dCTP was from ICN Biochemicals (Irvine). Oligotex-dT30 was obtained from Takara Shuzo Co., cDNAs for Cu/Zn-SOD and Mn-SOD was presented by Dr. Yoshikazu Tanaka (Suntory Co., Osaka) and by Dr. Ye-Shin Ho (Duke University Medical Center), respectively. All other reagents used were of analytical grade.

2.2. Animal experiments

Animals were subcutaneously injected with 10 IU of PMSG. After 48 h, ovulatory process was triggered by injecting 5 IU of hCG. At 0, 6, and 12 h after hCG injection, animals were killed by cervical dislocation. The abdomen was opened and both sides of ovariums, tubes and uterus were removed. Then, number of ova in the ampullary site of both tubes were counted.

2.3. Enzyme activity

After homogenization of the ovary in 3 vols. of phosphate-buffered saline (PBS), Triton X-100 was added to give a final concentration of 0.5%. After centrifugation at 100 000 \times g for 1 h, the supernatant fraction was obtained. To remove low molecular weight compounds,

Abbreviations: SOD, superoxide dismutase; PMSG, pregnant mare serum gonadotropin; hCG, human chorionic gonadotropin; SDS, sodium laurylsulfate; EDTA, ethylenediaminetetraacetic acid.

Correspondence address: E.F. Sato, Department of Medical Biology, Kochi Medical School, Nankoku-shi, Kochi 783, Japan. Fax: (81) (888) 66-6178.

the supernatant fraction was dialyzed against PBS for 60 min. Low molecular weight compounds were also removed by centrifugation through ultrafiltration membranes (Amicon PM-10) for 10 min. Under identical conditions, no significant activity of purified Mn-SOD was found to decrease. Cu/Zn-SOD and Mn-SOD activities in the dialyzed samples were determined at pH 7.8 by the xanthine oxidase-cytochrome *c* method in the presence or absence of 1 mM KCN and expressed as units/mg protein (U/mg) [12]. Protein concentration was determined by the method of Lowry using albumin as the standard [13].

2.4. Preparation of mRNA

Total RNA was isolated from the ovary according to the method of Chomczynsky and Sacchi [14]. Briefly, ovaries were homogenized for 1 min at full-speed using a Polytron homogenizer in a 5 ml solution containing 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% *N*-lauroylsarcosine and 100 mM 2-mercaptoethanol. To the homogenate was added 0.5 ml of 2 M sodium acetate (pH 4.0), 5 ml of water-saturated phenol and 1 ml of chloroform/3-methyl-1-butanol (24:1, v/v). The mixture was centrifuged at $3,000 \times g$ for 40 min at 4°C. The aqueous phase was mixed with 5 ml of 2-propanol and incubated at -20°C for at least 1 h. After centrifugation of the incubated mixture at $10,000 \times g$ for 10 min at 4°C, the precipitated RNA was dissolved in 700 μ l of guanidine thiocyanate solution, transferred into a 1.5 ml of polypropylene tube, and incubated with 1 vol. of 2-propanol at -20°C for 1 h. After centrifugation at $10,000 \times g$ for 10 min at 4°C, the precipitated RNA was resuspended in 75% ethanol, sedimented by centrifugation, and evacuated to dryness. Then, the precipitate was dissolved in 100 μ l of 0.5% sodium laurylsulfate (SDS). Polyadenylated poly(A)⁺ RNA was isolated by binding to Oligotex-dt30 [15]. Typically, 100 μ l of total RNA (150 μ g) was incubated in 100 μ l of 2% Oligotex-dt30 containing 10 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1% SDS at 65°C for 5 min and chilled on ice. After adding 20 μ l of 5 M NaCl, the mixture was incubated at 37°C for 10 min. Then, the mixture was centrifuged at $10,000 \times g$ for 15 min at room temperature and the supernatant fraction was removed. The pellet was suspended in 200 μ l of 0.1% diethyl pyrocarbonate-treated sterile H₂O, incubated at 65°C for 5 min, and chilled on ice. The incubated sample was centrifuged at $10,000 \times g$ for 15 min at room temperature. Then, the supernatant was added with 1/10 vol. of 3 M sodium acetate (pH 5.2) and 2.5 vols. of ethanol and incubated at -20°C for 3 h. After centrifugation at $10,000 \times g$ for 10 min at 4°C, the precipitated poly(A)⁺ RNA was resuspended in 75% ethanol, sedimented by centrifugation, and evacuated to dryness. Then, the precipitate was dissolved in 5 μ l of 0.5% SDS. Total RNA and poly(A)⁺ RNA concentration were estimated by their absorbance at 260 nm.

2.5. RNA blot analysis and Northern hybridization

Poly(A)⁺ RNA (3 μ g) was loaded on 1.2% MOPS/formaldehyde/agarose gel as described previously [16] and was electrophoresed for 3 h at 100 V. The electrophoresed RNA was transferred from the gel to a nylon membrane (Hybond-N, Amersham) using a vacuum blotting (VacuGene XL; Pharmacia-LKB) and cross-linked by UV irradiation.

cDNA probes for Cu/Zn-SOD, Mn-SOD, and β -actin were labeled with [α -³²P]dCTP (ICN; >3000 μ Ci/mmol) by Megaprime DNA labelling system (Amersham Japan, Tokyo). Hybridization was performed at 42°C for 12–16 h in a buffer containing 5 \times SSC (0.75 M NaCl, 75 mM Na-citrate, pH 7.0), 5 \times Denhart's solution (0.1% of each Ficoll-400, polyvinylpyrrolidone and bovine serum albumin), 0.5% SDS, 50% formamide and 0.02 mg/ml denatured salmon DNA. The membrane was washed with buffer A containing 2 \times SSC, 0.1% SDS at room temperature for 10 min, and two times with buffer B containing 0.1 \times SSC, 0.1% SDS at 65°C for 30 min. Hybridization signals were normalized to an internal standard (β -actin). They were subjected to autoradiography using Kodak X-AR film and an intensifying screen at -80°C. The autoradiography was analyzed with a Shimadzu CS-930 Densitometer.

3. RESULTS

3.1. Change in SOD activity during ovulation

To distinguish Cu/Zn- from Mn-type isozyme of SOD, the enzyme activities in the dialyzed supernatant of ovarian homogenates were measured in the presence or absence of KCN. Fig. 1 shows the change in ovarian activities of total, Cu/Zn- and Mn-type SOD during ovulatory process. With time after hCG treatment, total SOD activity decreased gradually from 17.56 ± 0.23 (at 0 h) to 13.08 ± 2.17 U/mg (at 12 h). In immature ovaries of control animals, the activity of Mn-SOD was equal to that of Cu/Zn-SOD. After stimulation by hCG, however, the activity of Mn-SOD started to decrease. In contrast, the activity of Cu/Zn-SOD decreased only slightly during the ovulatory process. Thus, the decrease in Mn-SOD activity was principally responsible for the decrease in total SOD activity of the ovary. The lowest activities of total and Mn-SOD were seen after 12 h of hCG treatment.

3.2. Expression of mRNAs for SOD isozymes

Fig. 2 shows the Northern blot analysis of ovarian SOD mRNA during the ovulatory process. The amount of Cu/Zn-SOD mRNA expression remained unchanged after hCG injection except for a transient decrease at 6 h after treatment. In contrast, the expression of Mn-SOD mRNA increased markedly with time and reached a maximum in 12 h after hCG treatment. Thus, the extent of Mn-SOD mRNA expression and the enzyme activity in the ovary did not correlate with each other.

3.3. Effect of SM-SOD on the ovulation

To know the role of superoxide radicals and SOD in the mechanism of ovulation, effect of a long-acting SOD (SM-SOD) was studied in hCG-treated rats. Fig. 3 shows the effect of SM-SOD on hCG-induced ovulation. Under control experimental conditions, the ovulation rate was 34.8 ± 0.8 ova/rat. The ovulation rate decreased significantly by intravenous administration of SM-SOD. The inhibition occurred dose-dependently. The maximum inhibition by SM-SOD was about 55%. The inhibitory action did not increase even if SM-SOD was increased up to 10 mg/kg. In contrast, H₂O₂-inactivated SM-SOD failed to inhibit the ovulation rate, suggesting that dismutation of superoxide radical per se was responsible for the inhibition of ovulation.

4. DISCUSSION

The present work demonstrates that ovarian levels of SOD activity, particularly that of Mn-SOD, markedly decreased in hCG-treated rats. This observation suggested that superoxide metabolism in and around mitochondria of ovarian cells changed significantly during the growth of follicles. Despite the marked decrease in Mn-SOD activity, ovarian levels of its mRNA increased

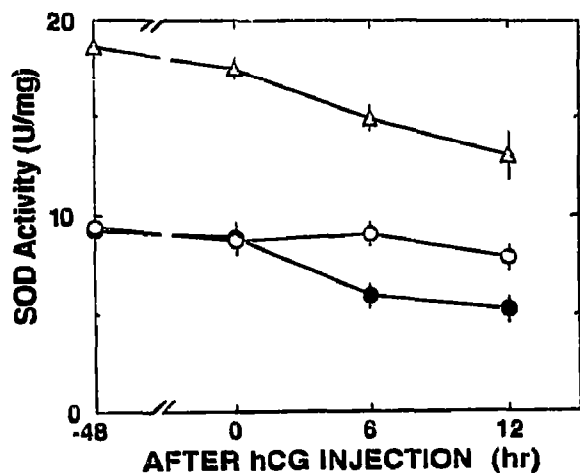


Fig. 1. hCG-induced changes in ovarian activity of SOD isozymes. The ovary was obtained from 22-day-old control rats. Ovaries were also obtained from hCG-treated rats at 0, 6 and 12 h after administration. SOD activities were measured as described under Materials and Methods. Data show mean \pm S.D. derived from the three animals. (Δ) total SOD; (\circ) Cu/Zn-SOD; (\bullet) Mn-SOD.

significantly after hCG treatment. These results might suggest that both rates of synthesis and degradation of Mn-SOD were increased in the ovary of hCG-treated

animals; the latter process occurred more rapidly than the former. Since Cu/Zn-SOD activity remained unchanged during the ovulatory process, the decrease in Mn-SOD activity might reflect preferential changes in mitochondrial status in the ovary. In fact, mitochondria in granulosa cells have been known to undergo swelling and reveal tubulo-vesicular cristae during the maturation of follicles [17]. Changes in mitochondrial properties and cell constituents during ovulation should be studied further.

The mechanism by which the rate of synthesis for Mn-SOD mRNA is enhanced remains to be studied. Recent studies revealed that ovarian cancer cells synthesized high levels of Mn-SOD and hence, plasma levels of this isozyme were increased in patients with ovarian cancer [18]. It should be noted that, in some cell lines, *de novo* synthesis of Mn-SOD is enhanced by various compounds involved in inflammatory reaction, such as tumor necrosis factor, interleukin-1, lipopolysaccharides, and phorbol myristate acetate [19–25]. It has been known that metabolic change similar to that found in inflammatory tissues also occurs in the ovary, which is prerequisite to the rupture of matured follicles [26]. Hence, a wide variety of nonsteroidal anti-inflammatory drugs, such as indomethacin, inhibited the ovula-

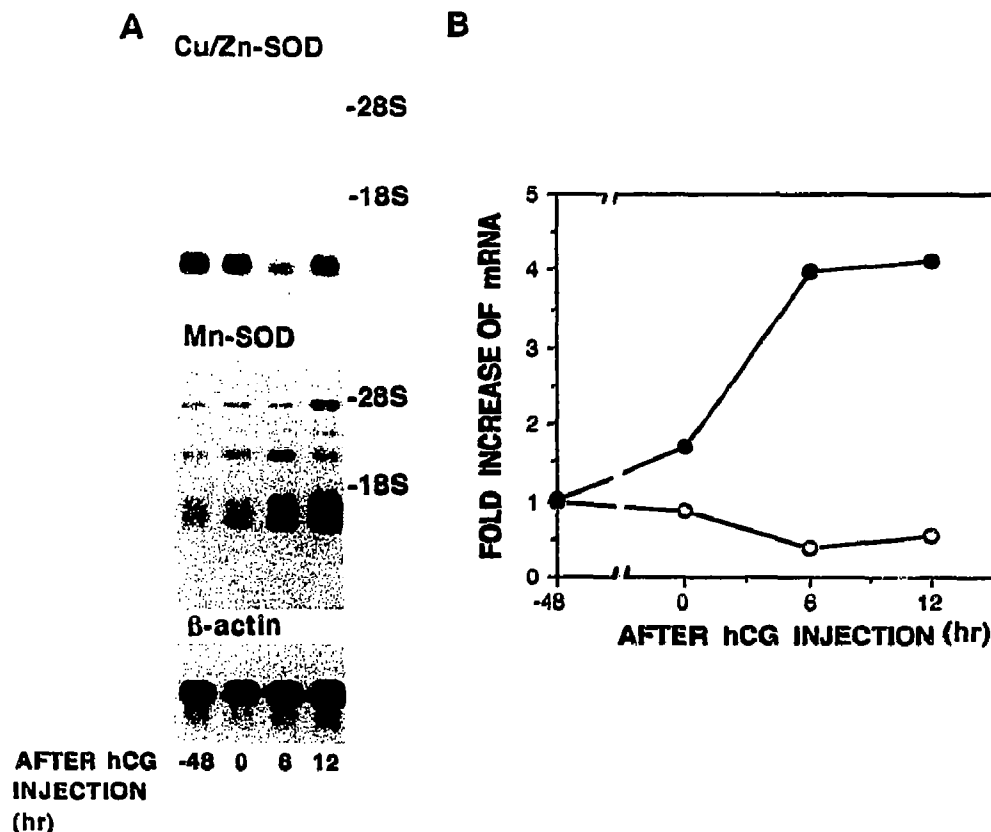


Fig. 2. Northern blot analysis of mRNA for Cu/Zn-SOD and Mn-SOD. Control ovaries (-48 h) were obtained from 22-day-old rats. Ovaries were also obtained from rats at 0, 6 and 12 h after hCG administration. Poly(A)⁺ RNA samples (3 μ g/lane) from these ovaries were electrophoresed, transferred to a nylon membrane, and hybridized as described under Materials and Methods. Densitometric analysis of the hybridization signals was performed using equal amounts of β -actin mRNA. (A) Northern blot analysis for Cu/Zn-SOD, Mn-SOD, and β -actin mRNAs. (B) Change in mRNA levels measured by densitometric analysis. Values represent the average of the duplicate samples. (\circ) Cu/Zn-SOD; (\bullet) Mn-SOD.

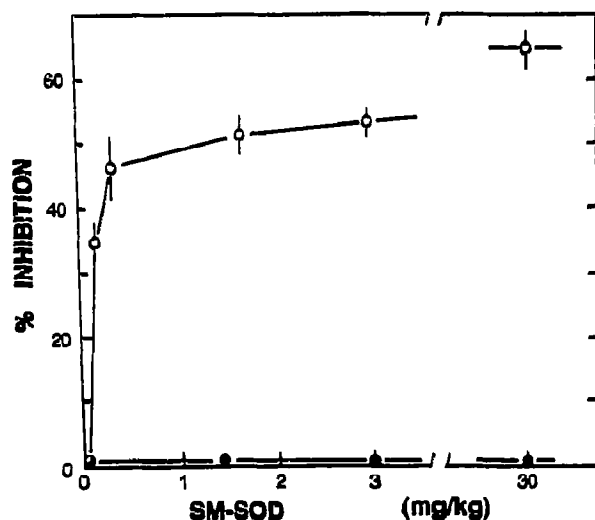


Fig. 3. Effect of SM-SOD and H_2O_2 -inactivated enzyme on the ovulation rate. Before 30 min of hCG-injection, animals were intravenously administered with either SM-SOD (○) or H_2O_2 -inactivated SM-SOD (●). After 12 h of hCG-injection, number of ova in the ampullary tubes were counted. Data show mean \pm S.D. derived from three animals.

tion [8]. These observations might suggest that metabolic perturbation that enhanced superoxide generation may possibly be involved in the mechanism for ovulation. In fact, significant amounts of macrophages accumulated in the growing follicles and promoted granulosa cell proliferation [27]. Furthermore, maturation of follicles in *op/op* mouse with marked reduction of macrophages in the ovary and other organs occurs extremely poorly [28]. These findings are not inconsistent with the notion that superoxide radicals generated by leukocytes may play important roles in the promotion of ovulatory process.

The present work also demonstrates that SM-SOD but not H_2O_2 -inactivated enzyme significantly inhibited the ovulation. Furthermore, subcutaneous administration of a large dose (30 mg/kg) of native SOD but not H_2O_2 -inactivated SOD also inhibited the ovulation by 40–50%. Recent study also revealed that SOD inhibited hCG-induced ovulation in an isolated perfused ovary of the rabbit [29]. Thus, the inhibition of hCG-induced ovulation by SM-SOD might principally be due to dismutation of superoxide radicals rather than nonspecific toxicity of the SM moiety.

SM-SOD circulates bound to albumin with half-life of 6 h, and accumulates in injured tissues whose pH is decreased [10,30]. Hence, SM-SOD effectively dismutates superoxide radicals in the circulation and around vascular endothelial cells of various tissues thereby decreasing oxidative stress [31,32]. SM-SOD also inhibited the increase in vascular permeability of injured tissues [33,34]. Preliminary experiments [35] revealed that vascular permeability of the ovary was markedly increased after hCG treatment by an SM-SOD-inhibitable mecha-

nism. These observations might suggest that superoxide radicals increased the vascular permeability of the ovary thereby promoting the disorganization of constituent cells in follicular walls, a prerequisite step to ovulation. The mechanism and the site(s) for superoxide generation in the ovary of hCG-treated animals should be studied further.

Acknowledgements: We gratefully acknowledge the gift of Cu/Zn-SOD cDNA from Dr. Yoshikazu Tanaka and Mn-SOD cDNA from Dr. Ye-Shin Ho. We thank Mrs. Taeko Okanishi for preparing the manuscript.

REFERENCES

- [1] Balentine, J.D. (1982) *Pathology of Oxygen Toxicity*, Academic Press, New York.
- [2] Burton, K.P., McCord, J.M. and Ghai, G. (1984) *Am. J. Physiol.* 246, 776–783.
- [3] Pederson, T.C. and Aust, S.D. (1973) *Biochem. Biophys. Res. Commun.* 52, 1071–1078.
- [4] Baldrige, C.W. and Gerard, R.W. (1933) *Am. J. Physiol.* 103, 235–236.
- [5] Armstrong, D.T. (1981) *J. Reprod. Fert.* 62, 283–291.
- [6] Kitani, H., Kobayashi, Y., Santulli, R., Wright, K.H. and Wallach, E.E. (1985) *Fert. Steril.* 43, 646–704.
- [7] Yoshimura, Y., Espey, L., Hosoi, Y., Adachi, T., Atlas, S.J., Ghodgaonkar, R.B., Dubin, N.H. and Wallach, E.E. (1988) *Endocrinology* 122, 2540–2546.
- [8] Espey, L.L., Stein, V.I. and Dumitrescu, J. (1982) *Fertil. Steril.* 38, 238–247.
- [9] Laloraya, M., Kumar, G.P. and Laloraya, M.M. (1988) *Biochem. Biophys. Res. Commun.* 57, 146–153.
- [10] Inoue, M., Ebashi, I., Watanabe, N. and Morino, Y. (1989) *Biochemistry* 28, 6619–6624.
- [11] Nakazono, K., Watanabe, N., Matsuno, K., Sasaki, J., Sato, T., and Inoue, M. (1991) *Proc. Natl. Acad. Sci. USA* 88, 10045–10048.
- [12] McCord, J.M. and Fridovich, I. (1969) *J. Biol. Chem.* 244, 6049–6055.
- [13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265–275.
- [14] Chomczynsky, P. and Sacchi, N. (1987) *Anal. Biochem.* 162, 156–159.
- [15] Kuribayashi, K., Hikata, M., Hiraoka, O., Miyamoto, C. and Furuichi, Y. (1988) *Nucleic Acids Res., Symposium Series* 19, 61–64.
- [16] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- [17] Sardul S. Guraya (1985) *Biology of Ovarian Follicles in Mammals*, pp. 221–227, Springer-Verlag.
- [18] Ishikawa, M., Yaginuma, Y., Hayashi, H., Shimizu, T., Endo, Y. and Taniguchi, N. (1990) *Cancer Res.* 50, 538–542.
- [19] Wong, G.H.W. and Goeddel, D.V. (1988) *Science* 242, 941–943.
- [20] Wong, G.H.W., Elwell, J.H., Oberley, L.W. and Goeddel, D.V. (1989) *Cell* 58, 923–931.
- [21] Visner, G.A., Dougall, W.C., Wilson, J.M., Burr, I.A. and Nick, H.S. (1990) *J. Biol. Chem.* 265, 2856–2864.
- [22] Kawaguchi, T., Takeyasu, A., Mastunobu, K., Uda, T., Ishizawa, M., Suzuki, K., Nishiura, T., Ishikawa, M. and Taniguchi, N. (1990) *Biochem. Biophys. Res. Commun.* 171, 1378–1386.
- [23] Tsan, M.-F., White, J.E., Treanor, C. and Shaffer, J.B. (1990) *Am. J. Physiol.* 259, L506–L512.
- [24] Masuda, A., Longo, D.L., Kobayashi, Y., Appella, E., Oppenheim, J.J. and Matsushima, K. (1988) *FASEB J.* 2, 3087–3091.

- [25] Fujii, J. and Taniguchi, N. (1991) *J. Biol. Chem.* 266, 23142–23146.
- [26] Espey, L.L. (1980) *Biol. Reprod.* 22, 73–106.
- [27] Fukumatsu, Y., Katabuchi, H., Naito, M., Takeya, M., Takahashi, K. and Okamura, H. (1992) *J. Reprod. Fertil.* (in press).
- [28] Naito, M., Hayashi, S., Yoshida, H., Nishikawa, S., Shultz, L. and Takahashi, K. (1991) *Am. J. Pathology* 139, 657–667.
- [29] Miyazaki, T., Sueoka, K., Dharmarajan, A.M., Atlas, S.J., Bulkley, G.B. and Wallach, E.E. (1991) *J. Reprod. Fert.* 91, 207–212.
- [30] Ogino, T., Inoue, M., Ando, Y., Awai, M., Meda, H. and Morino, Y. (1988) *Int. J. Protein Peptide Res.* 32, 153–159.
- [31] Watanabe, N., Inoue, M. and Morino, Y. (1989) *Biochem. Pharmacol.* 38, 3477–3483.
- [32] Kawamoto, S., Inoue, M., Tashiro, S., Morino, Y. and Miyauchi, M. (1989) *Arch. Biochem. Biophys.* 277, 160–165.
- [33] Ando, Y., Inoue, M., Morino, Y. and Araki, S. (1990) *Brain Res.* 477, 286–291.
- [34] Ando, Y., Inoue, M., Araki, Y. and Morino, Y. (1990) *Biochem. Biophys. Acta* 1073, 374–379.
- [35] Koyama, H., Inoue, M., Morino, Y., Ito, M. and Okamura, H. (1990) *Seikagaku* 62, 586.