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Nuclear Factor-κB Is Required for Tumor Necrosis Factor-α-Induced Manganese Superoxide Dismutase Expression in Human Endometrial Stromal Cells

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We recently found that manganese superoxide dismutase (Mn-SOD) is up-regulated by TNFα at the transcription level in human endometrial stromal cells (ESC) and that TNFα-induced Mn-SOD expression is mediated by protein kinase C (PKC)-dependent phosphorylation. This study was undertaken to investigate whether nuclear factor-κB (NF-κB), a transcription factor, is involved in Mn-SOD induction by TNFα or PKC in human ESC. Electrophoretic mobility shift assay revealed that TNFα (1 ng/ml) and phorbol 12-myristate 13-acetate (TPA; 0.4 μM), PKC activator, caused marked increases in nuclear NF-κB DNA binding activity. Secondly, ESC were incubated with MG132 (proteasome inhibitor) or SN50 (inhibitor of translocation of NF-κB into the nucleus) in the presence of TNFα or TPA. TNFα and TPA significantly increased Mn-SOD activities and Mn-SOD mRNA levels, and those effects were completely inhibited by MG132 and SN50. TNFα alone caused no effect on cell viability, but in the presence of MG132, TNFα significantly decreased cell viability. This inhibitory effect of MG132 was blocked by simultaneous addition of *N*-acetyl-L-cysteine, an antioxidant. In conclusion, the present study showed the involvement of NF-κB in Mn-SOD induction by TNFα or PKC in human ESC. This phenomenon could be a self-defense system of ESC against TNFα-mediated oxidative stress. (*J Clin Endocrinol Metab* 87: 3845–3850, 2002)

INFLAMMATORY CYTOKINES cause superoxide radical generation and damage cells (1, 2), whereas manganese superoxide dismutase (Mn-SOD), located in the mitochondria, protects cells by scavenging superoxide radicals. Protective roles of Mn-SOD against cytokine-mediated oxidative stress are well known from the finding that cytotoxic effects of cytokines can be reduced by increased levels of Mn-SOD (3–6). In addition, the importance of eliminating superoxide radicals from the mitochondria is illustrated by the neonatal death of mice lacking Mn-SOD expression (7, 8). Superoxide radicals and SOD have been reported to play important roles in the regulation of cellular function in the human endometrium (9–13). Very recently, we found that TNFα induced Mn-SOD expression in human endometrial stromal cells (ESC) (14). This phenomenon suggests an important self-defense system of ESC against TNFα-mediated oxidative stress, as ESC are exposed to TNFα from macrophages (15, 16), endometrial epithelial cells, and stromal cells (17–20), and receptors for TNFα are also expressed in ESC (21).

TNFα initiates not only an intracellular signaling pathway that leads to apoptosis through activation of caspase (22–24), but also a pathway to cell survival through activation of nuclear factor-κB (NF-κB), a transcription factor (25–28). However, the signaling pathway responsible for Mn-SOD induction by TNFα is still unclear in human ESC. We reported in human ESC that Mn-SOD mRNA was up-regulated by TNFα through a transcription mechanism that is not dependent on new protein synthesis (14). The Mn-SOD promoter has binding sites for NF-κB (29, 30). NF-κB has been proposed as a regulator for TNFα-induced gene expression in a variety of cell types (31), and there are some reports showing that NF-κB correlates with induction of Mn-SOD mRNA (29, 30, 32–34). Therefore, there is a possibility that NF-κB is involved in Mn-SOD induction by TNFα in human ESC. If this is true, it would strongly suggest that the phenomenon of Mn-SOD induction in response to TNFα is a self-defense system of ESC against TNFα-mediated cytotoxicity. We further examined the involvement of NF-κB in Mn-SOD induction by protein kinase C (PKC) activation, because we found that PKC-dependent phosphorylation mediated TNFα-induced Mn-SOD expression in human ESC (14).

Materials and Methods

This project was reviewed and approved by the committee of investigations involving human subjects of Yamaguchi University School of Medicine. Informed consent from the patient was obtained before collection of any tissue samples for this study.

Reagents

Phenol red-free DMEM and glutamine were purchased from ICN Biomedicals, Inc. (Aurora, OH). Streptomycin, penicillin, and 1× trypsin-EDTA were from Life Technologies, Inc. (Grand Island, NY). Collagenase, TNFα, phorbol 12-myristate 13-acetate (TPA), *N*-acetyl-L-cysteine, benzamidine, aprotinin, leupeptin, and phenylmethyl- sulfonylfluoride (PMSF) were obtained from Sigma (St. Louis, MO). SN50 and MG132 were obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). FCS was purchased from PAA Laboratories GmbH (Linz, Austria). Tissue flasks

Abbreviations: ESC, Endometrial stromal cells; IκB, inhibitor of NF-κB; Mn-SOD, manganese superoxide dismutase; NF-κB, nuclear factor-κB; PKC, protein kinase C; PMSF, phenylmethylsulfonylfluoride; TPA, phorbol 12-myristate 13-acetate.

and nylon mesh were from BD Biosciences (Franklin Lakes, NJ). Random hexamer and *Taq* DNA polymerase were obtained from Perkin-Elmer Co. (Foster City, CA). [α-^{32}P]Deoxy-CTP and [γ-^{32}P]ATP were purchased from Amersham (Arlington Heights, IL). Isogen was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

ESC isolation

Human endometrium was obtained at hysterectomy from patients with normal menstrual cycles, aged 40–49 yr, who underwent surgery for myoma uteri. Endometrial samples were histologically diagnosed as late proliferative phase according to published criteria (35). Tissue samples were washed with phenol red-free DMEM containing 4 mM glutamine, 50 μg/ml streptomycin, and 50 IU/ml penicillin and minced into small pieces of less than 1 mm^3. ESC were isolated as reported previously (10). In brief, after the enzymatic digestion of minced tissues with 0.2% collagenase in a shaking water bath for 2 h at 37 C, stromal cells were separated by filtration through a 70-μm pore size nylon mesh. The filtrates were washed three times with medium, and the number of viable cells was counted by trypan blue dye exclusion. The homogeneity of the stromal cell preparation was verified by immunocytochemistry using the specific antibody against stromal cells, vimentin (data not shown). Cells were seeded at 10^5 cells/cm^2 in 75-cm^2 tissue culture flasks and incubated in phenol red-free DMEM containing glutamine, antibiotics, and 10% dextran-coated charcoal-stripped fetal calf serum at 37 C in 95% air and 5% CO_2. After three passages (7–10 d after plating) by standard methods of trypsinization with 1× trypsin-EDTA, cells were distributed into 25-cm^2 tissue culture flasks. At more than 80% confluence (3–5 d after the distribution), cell culture medium was changed to treatment medium.

Cell culture

We first examined NF-κB activation by TNFα or TPA using electrophoretic mobility shift assay as described below. Cells were incubated with culture medium (serum-free phenol red-free DMEM supplemented with glutamine and antibiotics) containing TNFα (1 ng/ml) or TPA (0.4 μM) for 4 h at 37 C in 95% air and 5% CO_2. After incubation, cells were used for extraction of nuclear fraction. Secondly, we examined whether NF-κB is involved in Mn-SOD induction caused by TNFα or TPA. Activation of NF-κB is brought about by phosphorylation and ubiquitination of the inhibitor of NF-κB (IκB), followed by its degradation by the proteasome, thus allowing NF-κB translocation into the nucleus. We, therefore, tested the effects of MG132 (a proteasome inhibitor) or SN50 (an inhibitor of translocation of NF-κB into the nucleus) on Mn-SOD expression induced by TNFα or TPA. Cells were incubated with culture medium containing MG132 (3 μM) or SN50 (50 μg/ml) for 30 min and then stimulated with either TNFα (1 ng/ml) or TPA (0.4 μM) for 4 h (for mRNA analysis) or 8 h (for enzyme activity assay) at 37 C in 95% air and 5% CO_2. To examine cell viability, cells were seeded at 10^5 cells/ml into each well of a 24-well culture plate and incubated with or without MG132 in the presence of TNFα for 8 h. After incubation, cells were collected by pipetting carefully, and cell viability was tested by the trypan blue dye exclusion method. The concentrations of TNFα and TPA and the period of incubation were based on our previous report (14). Three different experiments were performed in triplicate.

Electrophoretic mobility shift assay

We obtained nuclear extracts using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Chemical Co., Rockford, IL). After incubation, cells were washed with PBS; resuspended in cytoplasmic extraction reagent supplemented with benzamidine (0.5 mg/ml), aprotinin (2 μg/ml), leupeptin (2 μg/ml), and PMSF (0.75 mM); and centrifuged at 16,000 × *g* for 5 min. The supernatant was removed, and the pellet was resuspended in nuclear extraction reagent supplemented with benzamidine (0.5 mg/ml), aprotinin (2 μg/ml), leupeptin (2 μg/ml), and PMSF (2 mM) and centrifuged at 16,000 × *g* for 10 min. The supernatant was concentrated by centrifugal filter devices (Microcon, Millipore Corp., Bedford, MA) and used as a nuclear fraction. The protein concentrations were approximately 2 mg/ml.

Electrophoretic mobility shift assay was performed using gel shift assay systems (Promega Corp., Madison, WI). NF-κB consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′; 5′-GCCTGGGAAAGTCCCCTCAACT-3′) were radiolabeled with [γ-^{32}P]ATP (3000 Ci/mmol, 10 mCi/ml) using T4 polynucleotide kinase. The labeled oligonucleotides was separated from unincorporated nucleotides by chromatography through a G-25 spin column (Amersham Pharmacia Biotech, Piscataway, NJ). The radiolabeled probe was incubated with 4 μg nuclear extracts prepared from untreated and TNFα- or TPA-treated cells in gel shift binding buffer. To verify the specificity of the binding reaction, an excess of unlabeled oligonucleotide was added to the reaction before adding the radiolabeled probe. The DNA-protein complexes were analyzed on 4% nondenaturing acrylamide gels.

SOD assay

After incubation, cells were washed with PBS, collected from three 25-cm^2 tissue culture flasks, resuspended in Tris-HCl buffer (0.01 M, pH 7.4), and sonicated. Mn-SOD activity was determined as reported previously (36). The amount of protein required for 50% inhibition in the absorbance at 550 nm was defined as 1 U (nitrite unit) of SOD activity. All data were expressed as nitrite units of SOD activity per milligram of protein. Protein concentrations were determined by a published method (37).

RT-PCR

Total RNA was isolated from the cultured cell with Isogen using the method provided by the manufacturer. For mRNA analysis, RT-PCR was performed with the oligonucleotide primers for Mn-SOD (5′-AGTTCAATGGTGGTGGTCATA-3′ and 5′-CAATCCCCAGCAGTGGAATAA-3′) as reported previously (10). Direct sequence analyses of the PCR products were performed for sequence verification (10). Two oligonucleotide primers (5′-CTGAAGGTCAAAGGGAATGTG-3′ and 5′-GGACAGAGTCTTGATGATCTC-3′) were also used to amplify ribosomal protein L19 as an internal control as reported previously (38). Briefly, 3 μg total RNA were reverse transcribed at 42 C in a reaction mixture (single-strength PCR buffer, 2.5 μM deoxynucleotide triphosphates, 5 μM random hexamer primer, 1.5 μM $MgCl_2$, and 200 IU Moloney murine leukemia virus reverse transcriptase). The RT product was divided into two equal aliquots (one tube was for L19 primers), and PCR was performed. For PCR amplification, a mixture containing the oligonucleotide primers (50 pmol), [α-^{32}P]deoxy-CTP (2 mCi at 3000 Ci/mmol), and *Taq* DNA polymerase (2.5 IU) was added to each reaction. Amplification was carried out for 25 cycles consisting of 95 C for 1 min, 54 C for 1 min, and 72 C for 1 min for Mn-SOD, followed by 10 min of final extension at 72 C in a programmed temperature control system PC-800 (ASTEC, Fukuoka, Japan). The predicted sizes of the PCR-amplified products were 282 bp for Mn-SOD and 194 bp for L19. A linear curve was plotted using the number of cycles of amplification (20, 25, 30, and 35 cycles) *vs.* densitometric values of the PCR products of Mn-SOD and L19 at each cycle, measured with a BAS2000 (Fuji Photo Film Co., Ltd., Tokyo, Japan). As linearity was obtained between 20 and 30 cycles for each primer of Mn-SOD and L19, the optimal number of cycles for amplification was determined to be 25 cycles (data not shown). Reaction products were electrophoresed on an 8% polyacrylamide nondenaturing gel. After autoradiography, band intensities were analyzed using a bioimaging analyzer BAS2000. For quantification, the density of the signal of Mn-SOD was normalized to that of the internal control L19.

Statistical analysis

Data were examined by ANOVA and Duncan's new multiple range test. Differences were considered significant at $P < 0.05$.

Results

As a first step in demonstrating the involvement of NF-κB in Mn-SOD induction by TNFα or TPA, nuclear NF-κB DNA binding activity in response to TNFα or TPA was examined, as NF-κB activates transcription after translocation into the nucleus. For this purpose, nuclear extracts were prepared

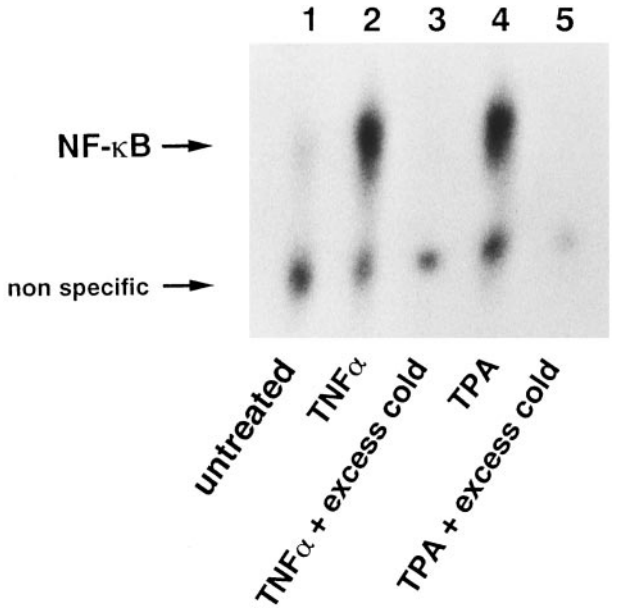

FIG. 1. Electrophoretic mobility shift assay of DNA-binding proteins from cells stimulated by TNFα or PK C activator (TPA). Radiolabeled NF-κB consensus oligonucleotides were incubated with nuclear extracts prepared from untreated and TNFα (1 ng/ml)- or TPA (0.4 μM)-treated human ESC. The DNA-protein complexes were analyzed on 4% nondenaturing acrylamide gels. Nuclear extracts from untreated cells (lane 1) showed negligible NF-κB-binding activity, whereas specific complexes were induced by TNFα (lane 2) or TPA (lane 4). To verify the specificity of the binding reaction, an excess of unlabeled oligonucleotide (excess cold) was added to the reaction before adding the radiolabeled probe. The complexes induced by TNFα or TPA were competed by addition of the excess cold (lanes 3 and 5, respectively).

from ESC treated with TNFα or TPA, and electrophoretic mobility shift assay was performed. As shown in Fig. 1, TNFα and TPA caused marked increases in NF-κB DNA-binding activity, whereas nuclear extracts from untreated cells showed negligible binding activity. To verify the specificity of the binding activity, an excess of unlabeled oligonucleotide was added to the reaction before adding the radiolabeled probe. Binding activities induced by TNFα or TPA were completely suppressed by the addition of unlabeled oligonucleotides.

Secondly, we examined whether induction of NF-κB by TNFα or TPA actually increases Mn-SOD expression. NF-κB is activated after the degradation of IκB by proteasome, a protease, followed by translocation of NF-κB into the nucleus. Therefore, ESC were incubated with MG132, an inhibitor of proteasome, in the presence of TNFα or TPA. TNFα significantly increased Mn-SOD activity and Mn-SOD mRNA level, and these stimulatory effects of TNFα were completely inhibited by MG132 (Fig. 2, A and B). In addition, the increases in Mn-SOD activities and Mn-SOD mRNA levels caused by TPA were also completely inhibited by MG132 (Fig. 2, C and 2D). MG132 alone had no effect on either activity or mRNA levels of Mn-SOD (Fig. 2). Furthermore, ESC were incubated with SN50, an inhibitor of translocation of NF-κB into the nucleus, in the presence of TNFα or TPA. TNFα and TPA significantly increased Mn-SOD mRNA levels, and these stimulatory effects of TNFα and TPA were completely inhibited by SN50 (Fig. 3). SN50 alone had no effect (Fig. 3).

As MG132 inhibited Mn-SOD induction by TNFα, we examined whether TNFα caused cell damage in the presence of MG132. As shown in Fig. 4, TNFα significantly decreased

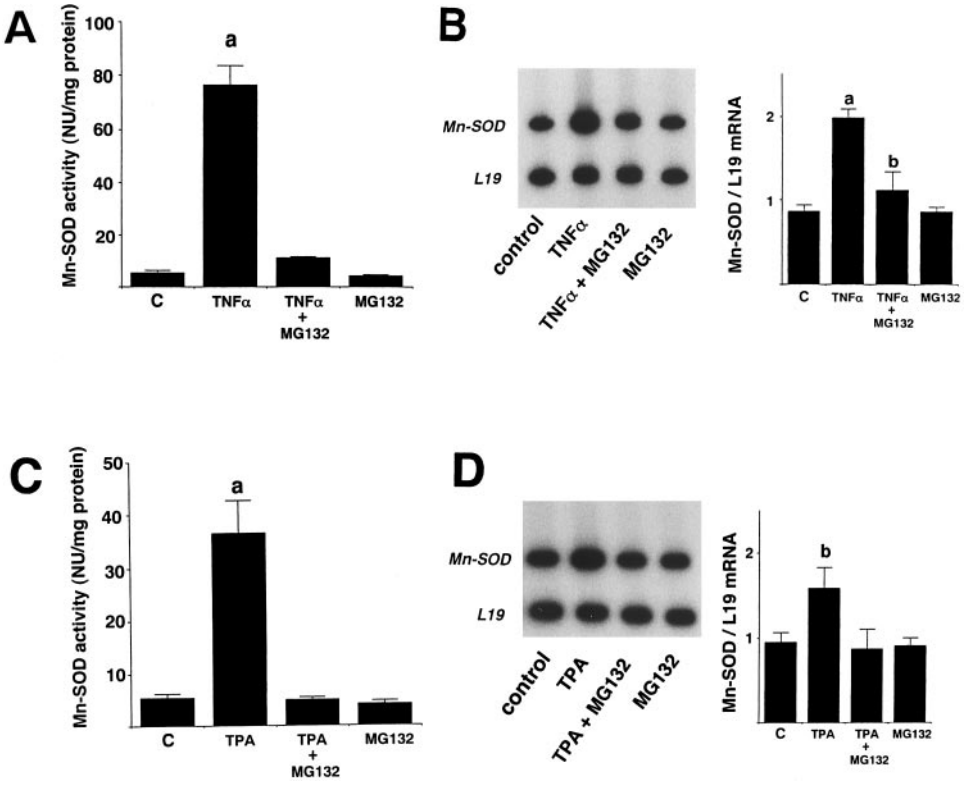

FIG. 2. Effects of MG132, an inhibitor of proteasome, on Mn-SOD expression induced by TNFα or PKC activator (TPA). Human ESC were incubated with culture medium containing MG132 (3 μM) for 30 min and then stimulated with TNFα (1 ng/ml) or TPA (0.4 μM) for 4 h (for mRNA analysis) or 8 h (for enzyme activity assay) at 37 C in 95% air and 5% CO_2. Mn-SOD activities are shown in A and C. Values are the mean ± SEM of three different experiments. mRNA expression was analyzed by RT-PCR and is shown in B and D. The intensity of the signals of Mn-SOD was normalized to that of the internal control L19. The quantification data (the ratio of Mn-SOD to L19) represent the mean ± SEM of three different experiments. A: a, $P < 0.01$ *vs.* the others. B: a, $P < 0.01$ *vs.* control; b; $P < 0.05$ *vs.* TNFα. C: a, $P < 0.01$ *vs.* the others. D: b, $P < 0.05$ *vs.* the others.

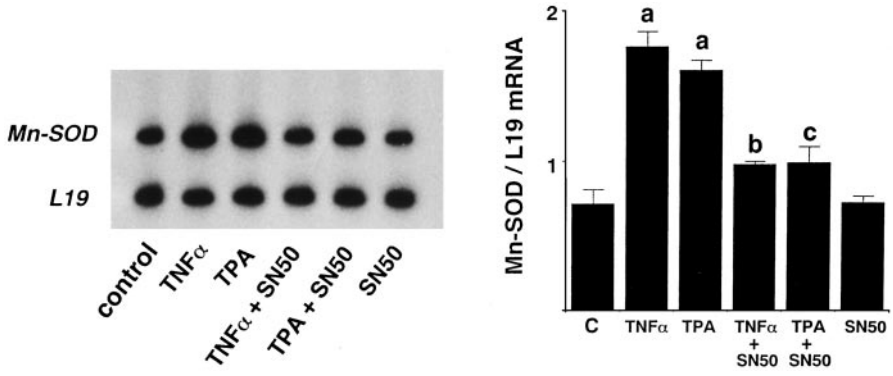

FIG. 3. Effects of SN50, an inhibitor of translocation of NF-κB into the nucleus, on Mn-SOD mRNA expression induced by TNFα or PKC activator (TPA). Human ESC were incubated with culture medium containing SN50 (50 μg/ml) for 30 min and then stimulated with either TNFα (1 ng/ml) or TPA (0.4 μM) for 4 h at 37 C in 95% air and 5% CO_2. mRNA expression was analyzed by RT-PCR. The intensity of the signals of Mn-SOD was normalized to that of the internal control L19. The quantification data (the ratio of Mn-SOD to L19) represent the mean ± SEM of three different experiments. a, $P < 0.01$ *vs.* control (C); b, $P < 0.01$ *vs.* TNFα; c, $P < 0.05$ *vs.* TPA.

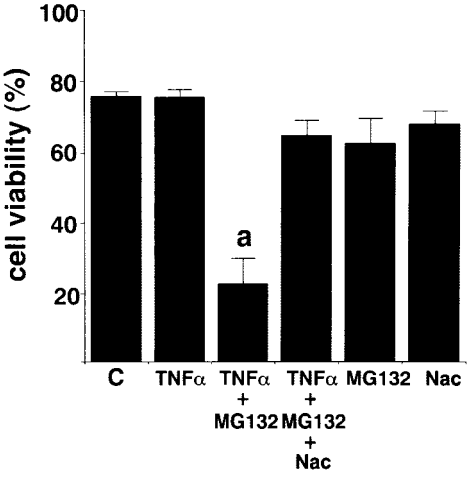

FIG. 4. Effects of TNFα on cell viability in the presence or absence of MG132. Human ESC were seeded at 10^5 cells/ml into each well of a 24-well culture plate; incubated in the presence of MG132 (3 μM) with or without *N*-acetyl-L-cysteine (Nac, 10 mM), an antioxidant, for 30 min; and then stimulated with TNFα (1 ng/ml) for 8 h at 37 C in 95% air and 5% CO_2. After incubation, cells were collected by careful pipetting, and cell viability was tested by the trypan blue dye exclusion method. Values are the mean ± SEM of three different experiments. a, $P < 0.05$ *vs.* the others.

cell viability in the presence of MG132, whereas TNFα alone caused no significant effect. The inhibitory effect of TNFα and MG132 on cell viability was blocked by simultaneous addition of *N*-acetyl-L-cysteine, an antioxidant. MG132 and *N*-acetyl-L-cysteine alone had no effect.

Discussion

We recently found in human ESC that Mn-SOD was upregulated by TNFα at a transcriptional level (14). The present study has demonstrated that TNFα-induced Mn-SOD expression is mediated through activation of NF-κB, a transcription factor. This study is, to our knowledge, the first report showing that NF-κB acts as a messenger in the signal transduction pathway of TNFα in human ESC, although recent reports have shown the presence of NF-κB signaling in the human endometrium (39–41). Accumulating data have shown that NF-κB mediates TNFα-induced Mn-SOD expression in a variety of cell types (29, 30, 32–34), and these reports may be supported by the finding that Mn-SOD promoter has binding sites for NF-κB (29, 30). NF-κB is retained in the cytoplasm by IκB in unstimulated cells. Upon cellular activation, IκB is phosphorylated by an IκB kinase complex, which targets IκB for ubiquitination and degradation and liberates NF-κB, resulting in nuclear translocation of NF-κB and activation of transcription. Thus, NF-κB-dependent activation does not require new protein synthesis. We reported that cycloheximide, which inhibits protein synthesis, did not block TNFα-induced Mn-SOD expression in human ESC (14). This result also suggests that Mn-SOD induction by TNFα is not dependent on new protein synthesis.

Regarding the specificity of SN50, SN50 blocked the nuclear import of other transcription factors, such as activating protein-1, which is involved in Mn-SOD induction by TNFα (42), when a high dose of SN50 (210 μg/ml) was used (43). However, a low dose SN50 (37.5 μg/ml) selectively inhibited NF-κB translocation (43). Although it is unclear how broadly the dose of SN50 (50 μg/ml) used in this study inhibits the nuclear import of other transcription factors, taking the data from the experiment with MG132 into consideration, the present study strongly suggests that NF-κB is involved in the Mn-SOD induction by TNFα.

Our previous study also showed that Mn-SOD induction by TNFα was mediated by PKC-dependent phosphorylation (14), which was consistent with the reports by Fujii *et al.* (44) and Suzuki *et al.* (45). The present study has further shown that PKC increases Mn-SOD expression through NF-κB activation in human ESC. As phosphorylation of IκB is one of the key steps leading to NF-κB activation, PKC may contribute to IκB phosphorylation. In fact, a number of reports have shown that PKC is involved in IκB phosphorylation (46–48). However, there is a possibility that NF-κB is activated by some messengers, *e.g.* reactive oxygen species, produced in response to PKC (32, 49, 50). On the other hand, there are some reports showing that activation of NF-κB by TNFα was independent of PKC (51, 52). These different reports may be due to the difference in cell types and circumstances of the cell.

Inflammatory cytokines such as TNFα cause cell damage through oxygen radical generation (1, 2). Interestingly, cells that are resistant to TNFα induce Mn-SOD to protect themselves from oxygen radical cytotoxicity by TNFα (3, 4). In this study TNFα caused no effect on cell viability, probably due to Mn-SOD induction, whereas the inhibition of Mn-SOD induction caused cell damage by oxygen radical generation. It is well known that TNFα can initiate an intracellular signaling pathway that leads to cell survival through NF-κB (25–28). Therefore, the present study strongly suggests that

Mn-SOD induction by TNFα via NF-κB activation is a self-defense system of human ESC against TNFα-mediated oxidative stress. This is consistent with several reports showing that the NF-κB and Mn-SOD system works for cell survival (29, 30, 32–34).

The present data suggest that some cells survive the inhibition of Mn-SOD induction despite low Mn-SOD activities because Mn-SOD activities in the TNFα plus MG132 group are probably derived from the surviving cells. It is difficult to clearly explain why these cells with low Mn-SOD activities are still alive. The Mn-SOD activity level of the surviving cells may have been sufficient to prevent cell death by oxygen radicals, because it has been suggested that cell death depends on the intracellular level of SOD activities as well as on cell type (53). These events may imply an intercellular variability or a heterogeneity, which is unavoidable.

A recent report has suggested that NF-κB signaling is activated in the human endometrium during the premenstrual phase and in early pregnancy (40). TNFα expression increases in the late secretory phase endometrium or gestational endometrium (17, 18, 21, 54). We reported that immunohistochemical expression of Mn-SOD increased in ESC during the late secretory phase and further increased in decidual cells of early pregnancy (9). Collectively, the observations from these *in vivo* studies may be supported by the *in vitro* data in this study showing that TNFα induces Mn-SOD expression through NF-κB activation in human ESC.

In conclusion, the present study has demonstrated that TNFα induces Mn-SOD expression through activation of NF-κB in human ESC. This phenomenon could be a self-defense mechanism of human ESC against oxygen radical cytotoxicity by TNFα and important for ESC to survive and contribute to successful pregnancy under the cytokine-rich environment of the human endometrium.

Acknowledgments

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