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QUARTERLY

A possible involvement of plasma membrane NAD(P)H oxidase in the switch mechanism of the cell death mode from apoptosis to necrosis in menadione-induced cell injury

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The effects of inhibitors of plasma membrane NADPH oxidase on menadione-induced cell injury processes were studied using human osteosarcoma 143B cells. The intracellular level of superoxide in the cells treated with menadione for 6 h reached a maximum followed by an abrupt decrease. The population of apoptotic cells detected by Annexin V and propidium iodide double staining also reached its maximum at 6 h of menadione-treatment while that of necrotic cells increased continuously reaching 90% of the total population at 9 h of the treatment. Pretreatment of the cells with inhibitors of NADPH oxidase, including diphenyliodonium chloride, apocynin, *N*-vanillylnonanamide and staurosporine was effective in lowering the menadione-induced elevations of superoxide, and also in the suppression of the switch of the cell death mode from apoptosis to necrosis in menadione-treated cells except for the case of staurosporine. These results strongly suggest that superoxide generated by NADPH oxidase, besides that generated by the mitochondria, may contribute to the remarkable increase in the intracellular level of superoxide in the cells treated with menadione for 6 h resulting in the switch from apoptosis to necrosis, although a direct evidence of the presence of active and inactive forms of NADPH oxidase in control and menadione-treated 143B cells is lacking at present.

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Abbreviations: AP, apocynin; DHE, dihydroethidium; DPI, diphenyliodonium chloride; FITC, fluorescein isothiocyanite; MEN, 2-methyl-1,4-naphthoquinone; *N*-VNA, *N*-vanillylnonanamide; PBS, phosphate-buffered saline; PI, propidium iodide; ROS, reactive oxygen species; STS, staurosporine.

Two types of cell death modes are known: apoptosis and necrosis. Apoptosis plays a key role in cell differentiation, organogenesis and various disease conditions. Apoptotic cells are phagocytosed by neighbouring cells, while necrotic cells cause local and general inflammatory reactions. There is a body of data in the literature demonstrating that a chemical at a low concentration may cause apoptosis, while at a higher concentration it will cause necrosis (Dive & Hickman, 1991; Eastman, 1993; Bonfoco *et al.*, 1995; Lieberthal *et al.*, 1996; Benchoua *et al.*, 2001). The detailed switch mechanisms involved in the transition of the cell death mode from apoptosis to necrosis remain to be solved. However, it is generally accepted that the intracellular level of ATP (Chandra *et al.*, 2000) and superoxide (Chandra *et al.*, 2000), and activities of caspases (Sun *et al.*, 1999) are three major components involved in the mechanism. Since apoptotic processes require energy, depletion of intracellular ATP results in the switch from apoptosis to necrosis. There are several experimental data to prove this (Eguchi *et al.*, 1997; Leist *et al.*, 1997; Ha & Synder, 1999). It is well established that reactive oxygen species (ROS) are intimately related to apoptosis and a burst in the intracellular level of ROS triggers the switch from apoptosis to necrosis (Bonfoco *et al.*, 1995; Nobel *et al.*, 1997; Hampton & Orrenius, 1997). It is also well established that a group of caspases play important roles in the apoptotic cascade and inactivation of caspases has been shown to trigger the switch from apoptosis to necrosis (Melino *et al.*, 1997; Lemaire *et al.*, 1998; Samali *et al.*, 1999).

Recently, we have found that menadione (2-methyl-1,4-naphthoquinone, MEN), a redox cycling agent, used as an anticancer drug, causes the switch from apoptosis to necrosis in human osteosarcoma cell line 143B cells (Kaminski *et al.*, 2003): the population of apoptotic cells reaches its maximum at 6 h after treatment with MEN at a concentration of 100 μM followed by abrupt decrease, while the population of necrotic cells increase continuously.

Mitochondria are the major source of superoxide. Recently, data have been accumulated to demonstrate that NADPH oxidase, originally detected in the plasma membrane of leukocytes, is localized also in various mammalian tissues: human placenta trophoblasts (Matsubara & Sato, 2001), vascular endothelial cells and vascular smooth muscle cells (Griendling *et al.*, 1994; 2000; Hohler *et al.*, 2000), human glomerular mesangial cells (Jones *et al.*, 1995), a hepatoma cell line (Ehleben *et al.*, 1997), and is involved in the apoptotic changes of the cell (Hu *et al.*, 2002; Kim *et al.*, 2002; Arroyo *et al.*, 2002).

The present study has been undertaken to find out if the superoxide generated by NADPH oxidase, besides that from the mitochondria, contributes to the burst in the intracellular level of ROS resulting in the transition of the cell death mode from apoptosis to necrosis in MEN-treated cells.

MATERIAL AND METHODS

Cell culture. Human osteosarcoma 143B cells (ATCC RL 8303) were cultured at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's Modified Eagles Medium (Nissui Co. Ltd., Tokyo, Japan) containing 1 mM pyruvate supplemented with 10% fetal bovine serum and 50 μg/ml kanamycin. The cells were kindly provided by Dr. M. Tanaka (Department of Gene Therapy, Gifu International Institute of Biotechnology, Yagi Memorial Park, Gifu, Japan).

Treatment of cells with various chemicals. Cells were cultured in the presence of chemicals specified below: MEN (final concentration, 100 μM; Sigma Chemicals Co., St. Louis, MO, U.S.A.); inhibitors of NADPH oxidase: diphenyliodonium chloride (DPI, final concentration, 1 μM; Sigma); apocynin (AP, final concentration, 500–1000 μM; Lan-

caster); *N*-vanillylnonanamide (*N*-VNA, final concentration, 125 μM; Sigma); staurosporine (STS, final concentration, 500 nM; Sigma). All reagents were prepared as 1000×-concentrated stock solutions in Me_2SO and stored at –20°C.

Detection of apoptotic and necrotic changes of the cell. Changes in phospholipid asymmetry of the plasma membrane and its intactness were detected using Annexin-V FITC Apoptosis Kit (BioSource International, Inc., Camarillo, CA, U.S.A.) composed of fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) double staining and subsequent flow cytometric analysis, essentially according to the method described by Gerasimenko *et al.* (2002) as described before (Kaminski *et al.*, 2003).

Cells growing in 6-cm culture dishes were collected by trypsinization, washed in phosphate-buffered saline (PBS), and collected with cells floating in the culture medium by centrifugation. Cells were suspended (1×10^6 cells/ml) in 100 μl AnnexinV-binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Then, FITC-Annexin V (5 μl/500 μl 1× AnnexinV-binding buffer) and PI (final concentration, 5 μg/ml) were added to the cell suspension and incubated for 30 min in the dark. All procedures were carried out at 22°C (Gerasimenko *et al.*, 2002). Samples were submitted to flow cytometer analysis using FACSCAN (Coulter, France).

Flow cytometric analysis of intracellular levels of oxygen free radicals. Overall intracellular superoxide generation was measured following the conversion of dihydroethidium (DHE) (Molecular Probes Inc.) into ethidium by the method of Mancini *et al.* (1997) as described before (Spodnik *et al.*, 2002). Cells growing in 6-cm culture dishes were stained with DHE (final concentration, 10 μM) for 30 min at 37°C in a humified atmosphere with 5% CO_2, washed in phosphate-buffered saline, re-suspended and submitted to FACS analysis.

Protein determination. Protein content was assayed using Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the manufacturer's instructions.

Statistical analysis. Data presented are the mean ±S.E. of at least three different experiments. Student's *t*-tests were used to evaluate significant differences. Values of the experimental groups were statistically different from those of the control at: a ($P < 0.001$), b ($0.001 < P < 0.01$), c ($0.01 < P < 0.02$), d ($0.02 < P < 0.05$).

RESULTS AND DISCUSSION

Recently, we have analyzed time-dependent changes of the cell injury processes induced by MEN (Kaminski *et al.*, 2003). We found that a population of apoptotic cells reaches its maximum at 6 h after the treatment followed by an abrupt decrease. On the other hand, the population of necrotic cells continued to increase during the course of the experiment for up to 24 h.

Since the intracellular level of ROS is known to be one of the triggering factors to induce the switch of the cell death mode, time-dependent changes of the intracellular level of superoxide in MEN-treated cells were analyzed (Fig. 1). The intracellular levels of superoxide began to increase at 2 h of MEN-treatment reaching its maximum at 6 h followed by an abrupt decrease. Since there are several reports in the literature to demonstrate that superoxide generated by plasma membrane NADPH oxidase is involved, besides that generated from the mitochondria, in the apoptotic processes in certain experimental conditions (Hu *et al.*, 2002; Kim *et al.*, 2002; Arroyo *et al.*, 2002), the effects of several inhibitors of NADPH oxidase on the MEN-induced changes in the intracellular level of superoxide were examined (Fig. 2A).

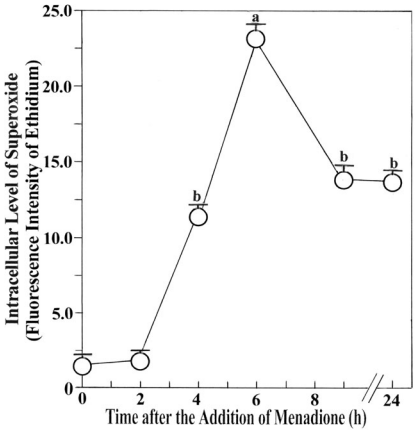

Figure 1. Time-dependent changes in the intracellular level of superoxide in MEN-treated 143B cells.

Cells were treated with 100 μM MEN for various lengths of time, collected, stained with dihydroethidium. The mean fluorescence intensity of ethidium per cell was plotted against the duration of MEN-treatment. Data are averages and standard error (mean ± S.E.) for five experiments. Values for the experimental groups are significantly different from those of the control (evaluated by Student's *t*-test) at: a ($P < 0.001$), b ($0.001 < P < 0.01$).

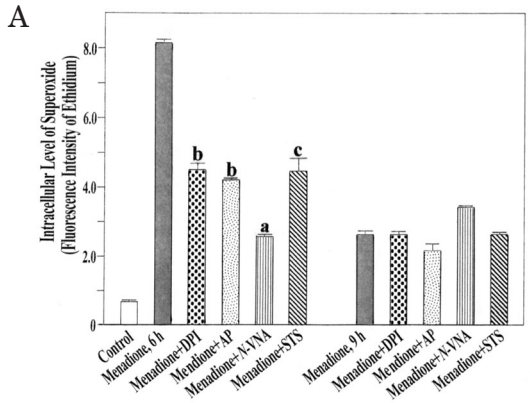

Figure 2. Effects of the pretreatment with inhibitors of NADPH oxidase on MEN-induced changes of the intracellular level of superoxide.

A. Cells were treated with MEN for 6 h or 9 h in the presence and absence of the pretreatment with inhibitors of NADPH oxidase indicated in the figure, and stained with dihydroethidium for the detection of the intracellular levels of superoxide. DPI: diphenyliodonium chloride; AP: apocynin; *N*-VNA: *N*-vanillylnonanamide; STS: staurosporine. Data are averages and standard error (mean ± S.E.) for three experiments. Comparisons were made between the data obtained from the cells treated with menadione alone for 6 h or 9 h and those treated with menadione in the presence of inhibitors of NADPH oxidase.

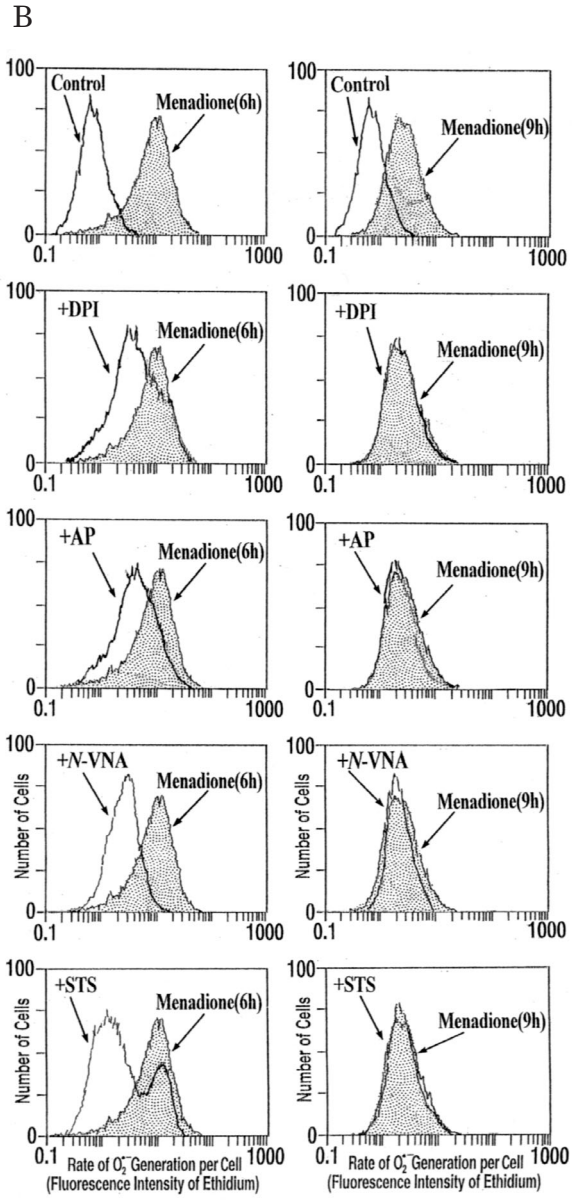

Figure 2. Continued.

B. Typical flow cytometric charts demonstrating intracellular levels of superoxide in cells treated with MEN in the absence and presence of the pretreatment with inhibitors of NADPH oxidase. Experimental conditions are the same as in Fig. 2A.

It is evident from the figure that the intracellular level of superoxide distinctly increased at 6 h of the MEN-treatment and the inhibitors of NADPH oxidase invariably suppressed this increase, although the intracellular level of superoxide remained higher than that of the control.

The intracellular level of superoxide in the cells treated with MEN for 9 h was distinctly lower than at 6 h and inhibitors of NADPH oxidase had practically no effect. Cells treated with MEN for 9 h were characterized ultrastructurally by condensed nuclei and swollen cytoplasm and thus could be designated as 'intermediate cells' indicating that they were in a transitional state from apoptosis to necrosis (Kaminski *et al.*, 2003).

Mitochondria in the intermediate cells were often extremely swollen with the rupture of the outer membrane. Furthermore, plasma membranes were sometimes ruptured as well. Thus it may be reasonable to assume that cells treated with MEN for 9 h were often seriously damaged so that the ability of mitochondria to synthesize ATP using molecular oxygen became decreased resulting in lowering of the rate of superoxide generation by mitochondria.

Typical examples of flow cytometric charts are shown in Fig. 2B. The peak intensity of ethidium in the cells treated with MEN for 6 h distinctly shifted to the right compared to that of control cells. The peak intensity of ethidium in the cells pretreated with various inhibitors of NADPH oxidase invariably shifted to the left compared to that of MEN-treated cells in the absence of the pretreatment with inhibitors of NADPH oxidase.

The peak intensity of the dye in the cells treated with MEN for 9 h also shifted to the right as in the case of cells treated with MEN for 6 h, but the degree of the shifts was much smaller. There were essentially no differences in the peak intensity of the dye between the cells treated with MEN in the presence and in the absence of the pretreatment with inhibitors of NADPH oxidase.

Next, the effects of pretreatment with inhibitors of NADPH oxidase on viability of MEN-treated cells were examined (Fig. 3). Cells were pretreated with inhibitors of NADPH oxidase and then treated with MEN for 6 h or 9 h. The cells were double-stained

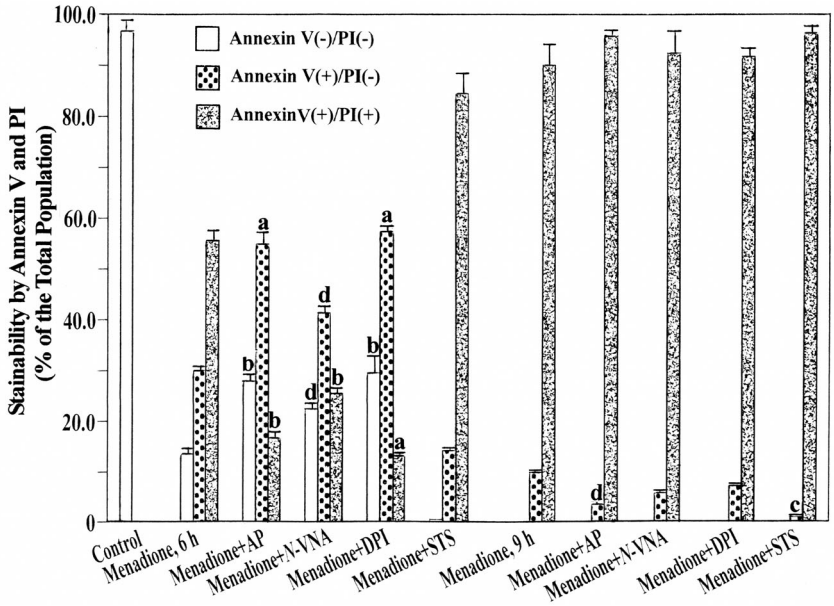

Figure 3. Viability of cells treated with MEN for 6 h or 9 h, in the presence and absence of the pretreatment with inhibitors of NADPH oxidase.

Cells treated with various experimental conditions were stained with Annexin V and PI for flow cytometric analysis of their viability. Annexin V (–)/ PI (–): viable cells; Annexin V (+)/ PI (–): apoptotic cells; Annexin V (+)/ PI (+): necrotic cells. Data are averages and standard error (mean ±S.E.) for three experiments. Comparisons were made between the data obtained for the cells treated with menadione alone for 6 h or 9 h and those treated with menadione in the presence of inhibitors of NADPH oxidase. Values of the latter are significantly different from those of the former at: a ($P < 0.001$), b ($0.001 < P < 0.01$), c ($0.01 < P < 0.02$), d ($0.02 < P < 0.05$).

with Annexin V and PI for viability. Cells were classified into three groups depending upon their stainability with the dyes: Annexin V(–)/PI(–), viable cells; Annexin V(+)/PI(–), apoptotic cells; and Annexin V(+)/PI(+), necrotic (intermediate) cells. At 6 h of the treatment with MEN, 30% of cells became apoptotic while 56% necrotic. On the other hand, pretreatment of cells with inhibitors of NADPH oxidase, except for the case of STS, distinctly suppressed the MEN-induced transition of the cell death mode from apoptosis to necrosis: the populations of apoptotic cells pretreated with AP, *N*-VNA, DPI were 55.0%, 41.4%, 57.4%, respectively. In the case of cells pretreated with STS, the population of apoptotic cells was even smaller (14.4%) than that of those treated with MEN alone with a distinct increase in the population of necrotic cells (84.6%). At 9 h of the MEN-treatment 90% of cells became necrotic, and pretreatment with NADPH oxidase inhibitors caused no change. Recently, we have shown that the major population of cells treated with MEN for 9 h become necrotic (intermediate cells), and mitochondria in these cells often become extremely swollen (Kaminski *et al.*, 2003). Thus it might be reasonable to assume that the ability of mitochondria in the cells treated with MEN for 9 h to use molecular oxygen for ATP synthesis is lowered compared to that of mitochondria in control cells. Although inhibitors of NADPH oxidase are partially effective in suppressing the transition of the cell death mode from apoptosis to necrosis, mitochondria are damaged when the incubation time with MEN exceeds 6 h and the cells become necrotic even in the presence of NADPH oxidase inhibitors.

Menadione has been shown to stimulate intracellular ROS generation *via* activation of NADPH oxidase from hepatocytes (Thor *et al.*, 1982), yeast (Yamashoji *et al.*, 1991) and a human B-lymphoma cell line and Jurkat cells (Suzuki & Ono, 1999). The present study strongly suggests a possible contribution of NADPH oxidase to the switch mechanism of the cell death mode from apoptosis to necrosis in MEN-treated human osteosarcoma 143B cells *via* the generation of superoxide. Detection of the intracellular localization of inactive and active forms of NADPH oxidase is currently under way and the results will be reported soon.

REFERENCES

Arroyo A, Modriancy M, Serinkan FB, Bello RI, Matsura T, Jiang J, Tyurin VA, Tyurina YY, Fadeel B, Kagan VE. (2002) NADPH oxidase-dependent oxidation and externalization of phosphatidylserine during apoptosis in Me_2SO-differentiated HL-60 cells. *J Biol Chem.*; **277**: 49965–75.

Benchoua A, Guégan C, Couriaud C, Hosseini H, Sampaïo N, Morin D, Onténiente B. (2001) Specific caspase pathways are activated in the two stages of cerebral infarction. *J Neuroscience.*; **21**: 7127–34.

Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA. (1995) Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci USA.*; **92**: 7162–6.

Chandra J, Samali A, Orrenius S. (2004) Triggering and modulation of apoptosis by oxidative stress. *Free Radic Biol Med.*; **29**: 323–33

Dive C, Hickman JA. (1991) Drug-target interactions: only the first step in the commitment to a programmed cell death? *Brit J Cancer.*; **64**: 192–6.

Eastman A. (1993) Apoptosis: a product of programmed and unprogrammed cell death. *Toxicol Appl Pharmacol.*; **121**: 160–4.

Eguchi Y, Shimizu S, Tsujimoto Y. (1997) Intracellular levels of ATP determine cell death fate by apoptosis or necrosis. *Cancer Res.*; **57**: 1835–40.

Ehleben W, Porwol T, Fandrey J, Kummer W, Acker H. (1997) Cobalt and desferroxamine

reveal crucial numbers of the oxygen sensing pathway in HepG2 cells. *Kidney Int.*; **51**: 483–91.

Gerasimenko JA, Gerasimenko OV, Palejwale A, Tepiki AV, Petersen OH, Watson AM. (2002) Menadione-induced apoptosis: role of cytosolic Ca^{2+} elevations and the mitochondrial permeability transition pore. *J Cell Sci.*; **115**: 485–97.

Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. (1994) Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res.*; **74**: 1141–8.

Griendling KK, Sorescu D, Ushio-Fukai M. (2000) NAD(P)H oxidase, role in cardiovascular biology and disease. *Circ Res.*; **86**: 494–501.

Ha HC, Synder SH. (1999) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. *Proc Natl Acad Sci USA.*; **96**: 13978–82.

Hampton MB, Orrenius S. (1997) Dual regulation of caspase activity by hydrogen peroxide: implication for apoptosis. *FEBS Lett.*; **414**: 552–6.

Hohler B, Holzapfel B, Kummer W. (2000) NADPH oxidase subunits and superoxide production in porcine pulmonary artery endothelial cells. *Histochem Cell Biol.*; **114**: 29–37.

Hu Q, Yu Z-X, Ferrans VJ, Takeda K, Irani K, Ziegelstein C. (2002) Critical role of NADPH oxidase-derived reactive oxygen species in generating Ca^{2+} oscillations in human aortic endothelial cells stimulated by histamine. *J Biol Chem.*; **277**: 32546–51.

Jones SA, Hancock JT, Jones OTG, Neubauer A, Topley N. (1995) The expression of NADPH oxidase components in human glomerular messangial cells: detection of protein and mRNA for p47-phos, p67-phos. *J Am Soc Nephrol.*; **5**: 1483–91.

Kaminski M, Masaoka M, Karbowski M, Kedzior J, Nishizawa Y, Usukura J, Wakabayashi T. (2003) Ultrastructural basis for the transition of cell death mode from apoptosis to necrosis in menadione-treated osteosarcoma 143B cells. *J Electron Microsc.*; **52**: 313–25.

Kim SH, Won SJ, Sohn S, Kwon HJ, Lee JY, Park JH, Gwag BJ. (2002) Brain-derived neurotrophic factor can act as a pronecrotic factor through transcriptional and translational activation of NADPH oxidase. *J Cell Biol.*; **159**: 821–31.

Leist M, Single B, Castaldi AF, Kuhle S, Nicotera P. (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med.*; **185**: 1481–6.

Lemaire C, Andreau K, Souvannavong V, Adam A. (1998) Inhibition of caspase activity induces a switch from apoptosis to necrosis. *FEBS Lett.*; **425**: 266–70.

Lieberthal W, Triaca V, Levine J. (1996) Mechanism of death induced by cisplatin in proximal tubular epithelial cells: apoptosis *vs* necrosis. *Am J Physiol.*; **270**: F700–8.

Mancini M, Anderson BO, Caldwell E, Sedghinasab M, Paty PB, Hockenbery DM. (1997) Mitochondrial proliferation and paradoxical membrane depolarization during terminal differentiation and apoptosis in human colon carcinoma cell line. *J Cell Biol.*; **138**: 449–69.

Matsubara S, Sato I. (2001) Enzyme histochemically detectable NAD(P)H oxidase in human placental trophoblasts: normal, preeclamptic, and fetal growth restriction-complicated pregnancy. *Histochem Cell Biol.*; **116**: 1–7.

Melino G, Bernassola F, Knight RA, Corasaniti MT, Mistico G, Finazzi-Agro A. (1997) S-nitrosylation regulates apoptosis. *Nature.*; **388**: 432–3.

Nobel CS, Burgess DH, Zhivotovsky B, Burkitt MJ, Orrenius S, Slater AF. (1997) Mechanism of dithiocarbamate inhibition of apoptosis: thiol oxidation by dithiocarbamate disulfides directly inhibits processing of the caspase-3 proenzyme. *Chem Res Toxicol.*; **10**: 636–43.

Samali A, Nordgren H, Zhivotovsky B, Peterson E, Orrenius S. (1999) A comparative study of apoptosis and necrosis in HepG2 cells: oxidant-induced caspase inactivation leads to necrosis. *Biochem Biophys Res Commun.*; **255**: 6–11.

Sun Y-L, Zhao Y, Hong X, Zhai Z-H. (1999) Cytochrome c release and caspase activation by menadione-induced apoptosis in plants. *FEBS Lett.*; **462**: 317–21.

Spodnik JH, Wozniak M, Budzko D, Teranishi M, Karbowki M, Nishizawa Y, Usukura J, Wakabayashi T. (2002) Mechanism of leflunomide-induced proliferation of mitochondria in mammalian cells. *Mitochondrion.*; **2**:163–79.

Suzuki Y, Ono Y. (1999) Involvement of reactive oxygen species produced via NADPH oxidase in tyrosine phosphorylation in human B- and T-lineage lymphoid cells. *Biochem Biophys Res Commun.*; **255**: 262–7.

Thor H, Smith MT, Hartzell P, Bellomo G, Jewell SA, Orrenius S. (1982) The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. *J Biol Chem.*; **257**: 12419–25.

Yamashoji S, Ikedo T, Yamashoji K. (1991) Extracellular generation of active oxygen species catalyzed by exogenous menadione in yeast cell suspension. *Biochim Biophys Acta.*; **1059**: 99–105.