Selective Involvement of Superoxide Anion, but Not Downstream Compounds Hydrogen Peroxide and Peroxynitrite, in Tumor Necrosis Factor-α-induced Apoptosis of Rat Mesangial Cells*

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Tumor necrosis factor-α (TNF-α) induces reactive oxygen species (ROS) that serve as second messengers for intracellular signaling. Currently, precise roles of individual ROS in the actions of TNF-α remain to be elucidated. In this report, we investigated the roles of superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and peroxynitrite (ONOO$^-$) in TNF-α-triggered apoptosis of mesangial cells. Mesangial cells stimulated by TNF-α produced O$_2^-$ and underwent apoptosis. The apoptosis was inhibited by transfection with manganese superoxide dismutase or treatment with a pharmacological scavenger of O$_2^-$, Tiron. In contrast, although exogenous H$_2$O$_2$ induced apoptosis, TNF-α-triggered apoptosis was not affected either by transfection with catalase cDNA or by treatment with catalase protein or glutathione ethyl ester. Similarly, although ONOO$^-$ precursor SIN-1 induced apoptosis, treatment with a scavenger of ONOO$^-$, uric acid, or an inhibitor of nitric oxide synthesis, N$^\text{3}$-nitro-l-argininemethyl ester hydrochloride, did not affect the TNF-α-triggered apoptosis. Like TNF-α-induced apoptosis, treatment with a O$_2^-$-releasing agent, pyrogallol, induced typical apoptosis even in the concurrent presence of scavengers for H$_2$O$_2$ and ONOO$^-$. These results suggested that, in mesangial cells, TNF-α induces apoptosis through selective ROS. O$_2^-$ but not H$_2$O$_2$ or ONOO$^-$, was identified as the crucial mediator for the TNF-α-initiated, apoptotic pathway.

Redox reactions regulate a broad array of signal transduction pathways. Reactive oxygen species (ROS), including superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO$^\cdot$), and peroxynitrite (ONOO$^-$) are now thought of as signaling molecules that are mobilized in response to stimuli. ROS modulate Ca$^{2+}$ signaling and protein phosphorylation events and, thereby, function as regulators for various biological processes, including gene expression, cell growth, differentiation, chemotaxis, and apoptosis (1).

ROS have been implicated in the signaling pathways initiated by tumor necrosis factor-α (TNF-α). Treatment of mammalian cells with TNF-α triggers generation of various ROS (2–5). Pharmacological experiments revealed that the mitochondrial respiratory chain is the major source of TNF-α-induced ROS (6, 7). Antioxidants inhibit various actions of TNF-α, e.g. activation of transcription factors, gene expression, and cytotoxicity, and externally added ROS mimic its biological potential (1, 8). These data support the current hypothesis that ROS serve as crucial second messengers for TNF-α signaling.

TNF-α induces apoptosis by engaging a cell surface receptor, TNF receptor 1 (TNFR1). Trimerization of TNFR1 by TNF-α associates an interaction of the receptors’ death domains. Subsequently, the adaptor protein, namely, TNFR-associated death domain (TRADD) binds to the clustered receptor death domains through its own death domain. TRADD functions as a platform adapter that recruits Fas-associated death domain (FADD). FADD contains a death effector domain that binds to an analogous domain within procaspase-8. Upon recruitment by FADD, oligomerization of procaspase-8 drives its activation via self-cleavage and subsequently activates downstream effector caspases, leading to apoptosis (9). During this signaling process, ROS are supposed to play a critical role. It is based on the following evidence. (i) Stimulation of cells with TNF-α results in a rapid rise in the levels of intracellular ROS. (ii) Addition of ROS or depletion of endogenous antioxidants induces cellular death. (iii) TNF-α-triggered cytotoxicity is inhibited by antioxidants/ROS scavengers, including thioredoxin, N-acetylcysteine, pyrrolidine dithiocarbamate, and superoxide dismutase (SOD) (8, 10, 11).

Oxygen normally accepts four electrons and is converted to water. In biological systems, however, partial reduction of oxygen occurs, resulting in the generation of cytotoxic ROS. That is, the sequential reduction of oxygen leads to the generation of O$_2^-$, H$_2$O$_2$, and OH$^-$ (12). O$_2^-$ and H$_2$O$_2$ are thought to be the primary species generated. O$_2^-$ also rapidly reacts with nitric oxide (NO), yielding another reactive species, ONOO$^-$ (13). In

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The abbreviations used are: ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; O$_2^-$, superoxide anion; H$_2$O$_2$, hydrogen peroxide; HO$^\cdot$, hydroxyl radical; ONOO$^-$, peroxynitrite; TNFR1, TNF receptor 1; TRADD, TNFR-associated death domain; FADD, Fas-associated death domain; SOD, superoxide dismutase; NF-$\kappa$B, nuclear factor-$\kappa$B; MCP-1, monocyte chemotractant protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SIN-1, 3-morpholinosydnonimine; GSH, glutathione ethyl ester; L-NAME, N$^3$-nitro-l-argininemethyl ester hydrochloride; Me$_3$SO, dimethyl sulfoxide; X-gal, 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside; ASK1, apoptosis signal-regulating kinase 1; IL-1β, interleukin 1β; FCS, fetal calf serum.
response to TNF-α, mammalian cells may produce O$_2^-$, H$_2$O$_2$, HO', and ONOO$^-$ (2–5). All of these ROS have the potential for triggering apoptosis (10, 12, 13). Currently, however, the involvement of and precise roles for individual ROS in mediating the apoptotic process are not well understood. It is largely unknown which compounds are required or not required for the TNF-α-initiated apoptosis. In the present report, we dissect the roles of ROS in the TNF-α signaling. The present data suggest that O$_2^-$ mediates TNF-α-induced apoptosis and that conversion of O$_2^-$ to its downstream ROS, including H$_2$O$_2$, ONOO$^-$, and HO' is not required to mediate the apoptotic process in mesangial cells.

EXPERIMENTAL PROCEDURES

Cells and Transfectants—Mesangial cells (SM43) were established from isolated glomeruli of a male Harlan Sprague-Dawley rat and identified as being of mesangial cell phenotype as described before (14). Medium containing 1% fetal calf serum (FCS) was used for experiments.

The nuclear factor-kB (NF-kB)–inactive mesangial cell line SM/IxBaM was created by overexpressing the super-repressor mutant of IxBa, IxBaM (15). SM/IxBaM cells produce IxBaM protein, which is resistant to degradation, and exhibit blunted activation of NF-kB in response to interleukin 1β (IL-1β) and TNF-α (16). As a control, mock-transfected mesangial cells that express neo alone (SMNeo) were used.

Northern Blot Analysis—Expression of IxBa, IxBaM, and monocyte chemoattractant protein 1 (MCP-1) was examined by Northern blot analysis (17). Confluent mesangial cells (SM43, SMNeo, and SM/IxBaM) cultured in the presence of 10% FCS were treated with or without human recombinant IL-1β (10 ng/ml; Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan) for 18 h and subjected to RNA extraction (18). cDNAs for IxBaM (19), J/E/MCP-1 (20), and glicer-aldehyde-3-phosphate dehydrogenase (GAPDH) (21) were labeled with [32P]dCTP and used for hybridization.

Pharmacological Manipulation—SM/IxBaM cells (1×10$^5$/well for 24-well plates; 5×10$^5$/well for 6-well plates) cultured in the presence of 1% FCS were stimulated with or without human recombinant TNF-α (250 units/ml; a gift from Dr. K. Noguchi, Teikyo University, Japan), H$_2$O$_2$ (100 μM; Sigma Immunochemicals, St. Louis, MO), or 3-morpholinosydnonimine (SIN-1, 1 mM; ICN Biomedicals, Oxford, UK) for up to 24 h. To examine the effects of antioxidants, cells were pretreated with catalase (500 units/ml; Sigma), glutathione ethyl ester (GSH, reduced form, 5 mM; Sigma), the cell-permeable O$_2^-$ scavenger Tiron (5 mM; Sigma) (22), ONOO$^-$ scavenger uric acid (1 mM; Sigma), the nitric oxide synthesis inhibitor Nω-nitro-argininemethyl ester hydrochloride (l-NNAME, 1 mM; Sigma), or dimethyl sulfoxide (Me$_2$SO; 20–100 μM; Sigma) for 1–3 h and then stimulated by TNF-α, H$_2$O$_2$, or SIN-1, as described above. The effect of O$_2^-$ on apoptosis was examined by exposing the cells to the superoxide-releasing agent 1,2,3-trihydroxybenzene (pyrogallol, 1 mM; Sigma) (23) for up to 14 h in the presence or absence of antioxi-dants (22).

Microscopic Analyses—Morphological examination was performed using phase-contrast microscopy. For fluorescence microscopy, cells were fixed with 4% formaldehyde in PBS for 10 min and stained using Hoechst 33258 (10 μg/ml; Sigma) for 1 h. Apoptosis was identified using morphological criteria, including shrinkage of the cytoplasm, membrane blebbing, and nuclear condensation and/or fragmentation. Quantitative assessment was performed using both attached and detached cells.

Ladder Detection Assay—After the induction of apoptosis, both attached and detached cells were harvested and subjected to ladder detection assay, as described previously (24).

Transfection—Using the calcium phosphate co-precipitation method (25), SM/IxBaM cells cultured in 24-well plates (1.0–2.0×10$^5$/well, 10% FCS) were co-transfected with pcI/meo-catalase (26), pcDNA3-Mn-SOD (11), or an empty plasmid pcDNA3 (Invitrogen, San Diego, CA) (500 ng/well, respectively) together with pcI-βGal (167 ng/well; a gift from Promega, Madison, WI). pcI-βGal introduces a β-galactosidase gene under the control of the immediate-early enhancer/promoter of human cytomegalovirus. After incubation overnight, the media was replaced with fresh medium containing 1% FCS. After 48 h, cells were treated with TNF-α (250 units/ml, 24 h) or H$_2$O$_2$ (100 μM, 6 h) and subjected to 5-bromo-4-chloro-3-indolyl β-D-galactopyrano- side (X-Gal) assay (27). Assays were performed in quadruplicate. The percentage of shrunken/rounded blue cells against the total number of blue cells was calculated for each well, and the mean value of four wells was used to compare data among different groups (28).

FIG. 1. Characterization of nuclear factor-kB (NF-kB)-inactive mesangial cells. NF-kB-inactive mesangial cells (SM/IxBaM) were created by overexpression of a super-repressor mutant of IxBa, IxBaM. As a control, parental SM43 mesangial cells (SM) and mock-transfected SM43 cells (SMNeo) were used. A, Northern blot analysis of endogenous IxBa and exogenous IxBaM. SM43, SMNeo, and SM/IxBaM were cultured in the absence (–) or presence (+) of IL-1β (10 ng/ml) for 18 h, and expression of IxBa and IxBaM was examined. Expression of GAPDH is shown at the bottom as a loading control. B, expression of a NF-kB-dependent gene, monocyte chemoattractant protein 1 (MCP-1), in SMNeo and SM/IxBaM in the absence or presence of IL-1 stimulation.

Assessment of O$_2^-$ Production—Production of O$_2^-$ was measured as the SOD-inhibitable reduction of cytochrome c (29). SM/IxBaM cells (2.5×10$^5$) were incubated for 3 h at 37 °C in physiological salt solution (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$, 2 mM CaCl$_2$, 1 mM MgCl$_2$, and 7.5 mM glucose; pH 7.4) containing 80 μM cytochrome c (Sigma) in the absence or presence of TNF-α (250 units/ml) and SOD (50 units/ml; Sigma). After the incubation, supernatants were collected and centrifuged, and the absorbance (550 nm) was measured using a spectrophotometer. Samples incubated in the absence of cells were used as blanks. O$_2^-$ production was expressed as nanomoles/10$^5$ cells (29). Assays were performed in quadruplicate.

Statistical Analysis—Data were expressed as means ± S.E. Statistical analysis was performed using the nonparametric Mann-Whitney U test to compare data among different groups. A p value of <0.05 was used to indicate a statistically significant difference.

RESULTS

Apoptosis of NF-kB-inactive Mesangial Cells in Response to TNF-α—In many cell types, TNF-α rarely triggers apoptosis because of induction of anti-apoptotic proteins via NF-kB-dependent mechanisms (30). TNF-α-sensitive mesangial cells were created by overexpression of a super-repressor mutant of IxBa, IxBaM (19). Expression of the transgene in the established SM/IxBaM was confirmed by Northern blot analysis (Fig. 1A). To examine whether the overexpressed IxBaM is functional, parental cells (SM), mock-transfected cells (SMNeo), and SM/IxBaM cells were cultured in the absence or presence of IL-1β (10 ng/ml), and expression of NF-kB-dependent genes, including endogenous IxBa and MCP-1 (31), was examined. Under the basal culture condition, only faint expression of IxBa and MCP-1 was observed. When stimulated by IL-1β, expression of these genes was markedly up-regulated in the control cells, SM and SMNeo. In contrast, the induction of IxBa and MCP-1 was attenuated in the NF-kB-inactive SM/IxBaM cells (Fig. 1, A and B).

Using the established cells, susceptibility to the TNF-α-induced apoptosis was examined. Confluent SMNeo cells and SM/IxBaM cells were exposed to TNF-α (250 units/ml), and microscopic analyses were performed. Phase-contrast microscopy showed that, like parental SM43 cells, SMNeo cells were resistant to TNF-α-induced cell death (Fig. 2A, left) at a high concentration (1000 units/ml, not shown). In contrast, TNF-α-induced shrinkage and detachment of SM/IxBaM cells (Fig. 2A, right). Staining of the cells with Hoechst 33258 exhibited condensation and fragmentation of nuclei in TNF-α-exposed SM/IxBaM cells (46%) but not in TNF-α-exposed SMNeo cells (1%) (Fig. 2B). Consistently, agarose gel electrophoresis detected dramatic DNA laddering in TNF-α-
SOD scavenges $O_2^\bullet$ by catalyzing the conversion of $O_2^\bullet$ to $H_2O_2$. The involvement of $O_2^\bullet$ in the TNF-α-initiated apoptosis was further confirmed by transient transfection. SM/IxBoM cells were co-transfected with an empty plasmid or an expression plasmid for Mn-SOD together with a β-galactosidase plasmid. Cells were then treated with TNF-α for 24 h and subjected to X-gal assay. As shown in Fig. 3D, treatment with TNF-α significantly increased percentages of apoptotic cells in mock-transfected cells (Mn-SOD (−)) (33.2 ± 6.4% (TNF-α) versus 10.4 ± 1.1% (untreated), p < 0.05). Transfection with Mn-SOD significantly reduced the percentage of apoptotic cells from 33.2 ± 6.4% in Mn-SOD (−)/TNF-α (−) to 15.8 ± 1.4% in Mn-SOD (+)/TNF-α (+).

To further confirm the involvement of $O_2^\bullet$ in the apoptotic process, production of $O_2^\bullet$ was examined in TNF-α-treated and -untreated cells. SM/IxBoM cells were incubated for 3 h with or without TNF-α (250 units/ml) and SOD (50 units/ml), and production of $O_2^\bullet$ was evaluated as the SOD-inhibitable reduction of cytochrome c. As shown in Fig. 3E, substantial induction of $O_2^\bullet$ was observed in the TNF-α-stimulated cells (10.4 ± 0.3 nmol/10^7 TNF-α-stimulated cells versus 4.8 ± 0.3 nmol/10^7 unstimulated cells, p < 0.05). Importantly, the reduction of cytochrome c in response to TNF-α was completely abrogated in the presence of SOD (not shown). This result indicated that $O_2^\bullet$ but not other ROS with reducing activity, was produced by mesangial cells in response to TNF-α.

Role of $H_2O_2$ in the TNF-α-induced Apoptosis—In the biological systems, $H_2O_2$ is primarily produced from $O_2$ or generated from $O_2^\bullet$ via SOD. To examine the role of $H_2O_2$ in the TNF-α-induced apoptosis of mesangial cells, SM/IxBoM cells were pretreated with cell-permeable scavengers of $H_2O_2$, GSH (5 mM) and catalase (500 units/ml), and $O_2^\bullet$ was evaluated as the SOD-inhibitable reduction of catalase. As shown in Fig. 3F, substantial induction of $O_2^\bullet$ was observed in the TNF-α-stimulated cells (10.4 ± 0.3 nmol/10^7 TNF-α-stimulated cells versus 4.8 ± 0.3 nmol/10^7 unstimulated cells, p < 0.05). Importantly, the reduction of catalase in response to TNF-α was completely abrogated in the presence of SOD (not shown). This result indicated that $O_2^\bullet$ but not other ROS with reducing activity, was produced by mesangial cells in response to TNF-α.

Role of ONOO− in the TNF-α-induced Apoptosis—ONOO− rapidly reacts with NO, yielding another reactive species, ONOO− (13). TNF-α is known to induce production of NO as well as $O_2^\bullet$, leading to generation of ONOO− (5). Recent investigations have suggested that, like $O_2^\bullet$ and $H_2O_2$, ONOO− has the ability to induce apoptosis (13). To examine the role of ONOO− in the TNF-α-initiated apoptosis of mesangial cells, SM/IxBoM cells were pretreated with a scavenger of ONOO−, uric acid (1 mM).
Selective Involvement of $O_2^*$ in TNF-α-induced Apoptosis

Fig. 3. Role of $O_2^*$ in the TNF-α-induced apoptosis. A–C, SM/IxBaM cells were pretreated with (+) or without (−) the cell-permeable $O_2^*$ scavenger, Tiron (5 mm), and stimulated by TNF-α (250 units/ml) for 20 h. A, microscopic analyses. Top, phase-contrast microscopy; bottom, Hoechst staining. B, quantitative assessment of apoptosis. After Hoechst staining, percentages of apoptotic cells with condensed and/or fragmented nuclei were calculated. The asterisk indicates a statistically significant difference (p < 0.05). C, DNA ladder assay. D, transient transfection with manganese superoxide dismutase (MnSOD). SM/IxBaM cells were co-transfected with an empty plasmid pcDNA3 (Mn-SOD (−)) or pcDNA3-MnSOD (Mn-SOD (+)) together with pCIBgal, which introduces a β-galactosidase gene. Cells were then treated with (+) or without (−) TNF-α (250 units/ml, 24 h) and subjected to 5-bromo-4-chloro-3-indolyl β-D-galactopiranoside (X-gal) assay. Percentage of shrunk/rounded blue cells against total blue cells was calculated for each well, and the mean value of four wells was used to compare data among different groups. Assays were performed in quadruplicate. The asterisk indicates a statistically significant difference (p < 0.05). NS, not significant. E, Assessment of $O_2^*$ production. SM/IxBaM cells were incubated for 3 h in the presence of 80 µM cytochrome c with or without TNF-α (250 units/ml) and SOD (50 units/ml). Production of $O_2^*$ was measured as the SOD-inhibitable reduction of cytochrome c. $O_2^*$ production was expressed as nanomoles per 10^6 cells. Assays were performed in quadruplicate. The asterisk indicates a statistically significant difference (p < 0.05).

or an inhibitor of nitric oxide synthesis, l-NAME (1 mM), and stimulated by TNF-α. Stimulation with the ONOO− precursor SIN-1 (1 mM) was used as a positive control. SIN-1 simultaneously generates NO and $O_2^*$, forming ONOO−. Exposure of the cells to SIN-1 induced round-up of the cells, as shown in Fig. 5A (bottom). Hoechst staining showed condensation and fragmentation of nuclei typical of apoptosis (Fig. 5B, bottom). Pretreatment with uric acid abolished the SIN-1-induced morphological changes. In contrast, under the same experimental condition, TNF-α-induced apoptosis was not affected by uric acid or l-NAME (Fig. 5, A and B, top). The percentages of apoptotic cells were 0.2 ± 0.2% in untreated control, 7.5 ± 0.7% in TNF-α alone, 9.8 ± 1.3% in uric acid + TNF-α, and 6.6 ± 0.9% in l-NAME + TNF-α (not statistically different) (Fig. 5C).

The lack of involvement of $H_2O_2$ and ONOO− was further confirmed by concurrent scavenging of these ROS. SM/IxBaM cells were pretreated with uric acid together with catalase and stimulated by TNF-α, $H_2O_2$, or SIN-1. Phase-contrast microscopy and Hoechst staining showed that the double ROS scavenging attenuated apoptosis induced by $H_2O_2$ or SIN-1. However, the TNF-α-induced apoptosis was not inhibited even in the concurrent presence of the $H_2O_2$ scavenger and the ONOO− scavenger (Fig. 6, A and B). The percentages of apoptotic cells were 0.6 ± 0.2% in untreated control, 18.9 ± 2.9% in TNF-α alone, and 27.3 ± 3.0% in uric acid/catalase + TNF-α (Fig. 6C). Consistently, TNF-α-triggered DNA laddering was observed regardless of the presence of uric acid and catalase (Fig. 6D).

HO· is generated by reduction of $H_2O_2$ or through ONOO− (12). The lack of involvement of $H_2O_2$ and ONOO− in the TNF-α-induced apoptosis was further confirmed using a HO· scavenger, Me$_3$SO. SM/IxBaM cells were pretreated with Me$_3$SO (20–100 mM) and stimulated by TNF-α. As shown in Fig. 6E, pretreatment with Me$_3$SO did not affect the TNF-α-induced apoptosis. The percentages of apoptotic cells were 1.2 ± 0.5% in untreated control, 19.5 ± 2.1% in TNF-α alone, 17.8 ± 1.1% in 20 mM Me$_3$SO + TNF-α, and 17.7 ± 1.1% in 100 mM Me$_3$SO + TNF-α (not statistically different).

Induction of Apoptosis by $O_2^*$ without Its Conversion to Downstream ROS—To evaluate the pro-apoptotic potential of $O_2^*$, SM/IxBaM cells were stimulated by $O_2^*$-releasing agent, pyrogallol. Hoechst staining showed that pyrogallol induced round-up of the cells and condensation and fragmentation of nuclei typical of apoptosis (Fig. 7A, left). The percentage of apoptotic cells was increased from 0.6 ± 0.2% to 37.8 ± 5.3% by the treatment with pyrogallol (Fig. 7A, right).

To examine whether $O_2^*$ induces apoptosis even without its conversion to $H_2O_2$ or ONOO−, SM/IxBaM cells were stimulated by pyrogallol in the presence of excessive catalase (1000 units/ml) and uric acid (2 mM). Hoechst staining showed that substantial induction of apoptosis was still observed even in the concurrent presence of scavengers for $H_2O_2$ and ONOO− (Fig. 7B, left). The percentages of apoptotic cells were 0.3 ± 0.3% in uric acid/catalase and 11.3 ± 1.1% in uric acid/catalase + TNF-α (Fig. 7B, right).
DISCUSSION

TNF-α induces generation of ROS that may serve as second messengers for cell death signaling. Currently, precise roles of individual ROS in the cytotoxic action of TNF-α are not well understood. Previous reports showed that cellular sensitivity or resistance to TNF cytotoxicity is correlated with decreased or increased levels of SOD, respectively (11, 32, 33). These data indicated that O$_2^-$ has a role in mediating TNF-induced cellular death. However, TNF-α induces both necrosis and apoptosis via ROS-dependent mechanisms (6, 7, 34, 35). Current unanswered questions are; (i) whether or not O$_2^-$ is indeed essential for TNF-α-induced apoptosis, and if so, (ii) how the secondary compounds downstream of O$_2^-$ contribute to the apoptotic process. In this report, we provided evidence for the selective, crucial role of ROS in the TNF-α-triggered apoptosis of mesangial cells.

In biological systems, O$_2^-$ is rapidly reduced by SOD to H$_2$O$_2$. O$_2^-$ also rapidly reacts with NO, yielding ONOO$^-$ (13). Once O$_2^-$ is generated in response to TNF-α, H$_2$O$_2$ and ONOO$^-$ may be subsequently produced and contribute to the apoptotic process. In the present study, however, scavengers of H$_2$O$_2$ and ONOO$^-$ did not have any effects on TNF-α-induced apoptosis. This evidence supports the selective and differential roles of ROS in mediating the apoptotic pathway. Only O$_2^-$ is necessary, and conversion of O$_2^-$ to its downstream compounds H$_2$O$_2$ and ONOO$^-$ is supposedly not required to mediate the TNF-initiated apoptosis.

HO$^+$ is generated by reduction of H$_2$O$_2$ or through ONOO$^-$ (12). HO$^+$ is known to be a highly reactive ROS and may be responsible for the oxidative damage of the cells (12). A previous report showed that induction of HO$^+$ is responsible for the cytotoxic effect of TNF-α on tumor cells (4). However, based on our current data, the contribution of HO$^+$ to the apoptotic proc-
The selective involvement of $O_2^-$ in TNF-α-induced apoptosis by mesangial cells is unlikely, because: (i) TNF-α-induced apoptosis occurred independently of $H_2O_2$ and $ONOO^-$, the compounds upstream of $HO^-$; and (ii) the scavenger of $HO^-$, Me$_2$SO, did not affect TNF-α-induced apoptosis.

Intracellular sources of ROS include mitochondrial oxidation, the microsomal cytochrome P-450 system, and plasma membrane NADPH oxidases. Previous studies suggested that the mitochondrial respiratory chain is the major source of TNF-induced formation of ROS (6, 7). In mesangial cells, the NADPH oxidase system seems to play a crucial role in the

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**Fig. 6. Induction of apoptosis by TNF-α in the concurrent presence of scavengers for $H_2O_2$ and $ONOO^-$.** SM/IbBaM cells were concurrently pretreated with uric acid (1 mM) and catalase (500 units/ml) and stimulated by TNF-α (250 units/ml). Stimulation with $H_2O_2$ (100 μM) and SIN-1 (1 mM) was used as positive controls. A, phase-contrast microscopy. B, Hoechst staining. C, quantitative assessment of apoptosis. Asterisks indicate statistically significant differences ($p < 0.05$). NS, not significant. D, DNA ladder assay. E, SM/IbBaM cells were pretreated with or without Me$_2$SO (0–100 mM) and stimulated by TNF-α. Percentages of apoptotic cells were evaluated by Hoechst staining. NS, not significant.
generation of ROS. For example, mesangial cells have functional NADPH oxidase components (36), and inhibition of the NADPH oxidase system suppresses expression of chemokines in response to TNF-α (37). The NADPH oxidase system may participate in the apoptotic process mediated by \( \text{O}_2^- \) in TNF-α-stimulated mesangial cells.

In contrast to the knowledge on the sources of ROS, little is understood about molecular targets of ROS during the apoptotic process. Direct DNA damage or formation of oxidized lipids in cell membranes may mediate or facilitate TNF-α-induced apoptosis (10). Alternatively, particular signaling molecules may be affected by ROS directly or indirectly. A recent study suggested that apoptosis signal-regulating kinase 1 (ASK1) is a possible target of ROS (38). ASK1 is a member of the mitogen-activated protein kinase kinase superfamily that activates both the c-Jun N-terminal kinase pathway and the p38 MAP kinase pathway by direct phosphorylation of MKK3, -4, -6, and -7 (39, 40). ASK1 is involved in the TNF-α-induced apoptotic pathway, because ASK1 is activated by TNF-α in many cell types, overexpression of ASK1 induces apoptosis, and expression of dominant-negative ASK1 inhibits TNF-α-induced apoptosis (40). Gotoh et al. (38) found that ROS induces activation of ASK1, that TNF-α-triggered activation of ASK1 is inhibited by antioxidants, and that ROS-induced apoptosis is markedly enhanced by overexpression of ASK1. These results suggested that TNF-α-induced activation of ASK1 is caused by ROS and contributes to the induction of apoptosis. Of note, ASK1 has a cysteine-rich domain in its N terminus. It might be a direct target for the action of ROS (38).

TNF-α induces apoptosis by engaging TNFR1. After ligation of the receptors, TRADD and FADD are recruited, leading to activation of downstream effector caspases (9). A recent report showed that activation of Z-VAD-sensitive caspase(s) is required for mitochondria-dependent ROS production (41). This result is consistent with another recent report that showed that staurosporine-induced apoptosis involves caspase-1-like proteases as initiators upstream of \( \text{O}_2^- \) production (42). These data indicate that the ROS-mediated process is an event downstream of serial caspase activation initiated by TNF-α.

Another putative mechanism implicated in TNF-α-mediated apoptosis is via generation of ceramide, the hydrolyzed product of the phospholipid sphingomyelin (43). A recent study suggested that the sphingomyelin pathway has a role in TNF-α-induced ROS production. Garcia-Ruiz et al. showed that mitochondria isolated from TNF-treated cells exhibit an increase in the amount of ceramide, that addition of C2-ceramide to mitochondria leads ROS generation, and that blockade of the electron transport chain prevents the C2-ceramide-induced production of ROS (3). In contrast, another recent study indicated that ROS participate in TNF-mediated ceramide production (44), suggesting that the ROS-sensitive pathway may be located upstream of ceramide production. Further investigation is required to disclose the relationship between ROS and the sphingomyelin pathway during TNF-α-induced apoptosis.

The differential roles of ROS in signal transduction pathways, especially in apoptotic pathways, have not been addressed before. The present study showed the selective involvement of \( \text{O}_2^- \) but not its downstream compounds \( \text{H}_2\text{O}_2 \), ONOO−, and HO· in TNF-α-induced apoptosis. To our knowledge, this is the first study to demonstrate the differential roles of \( \text{O}_2^- \) and downstream compounds in this particular apoptotic process.

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Selective Involvement of Superoxide Anion, but Not Downstream Compounds Hydrogen Peroxide and Peroxynitrite, in Tumor Necrosis Factor-α-induced Apoptosis of Rat Mesangial Cells

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