Previously we showed that rat mesangial cells are normally resistant to tumor necrosis factor-α (TNF-α)-induced apoptosis. They are made susceptible to the apoptotic effect of TNF-α when pretreated with actinomycin D, cycloheximide or vanadate. A sustained c-Jun N-terminal protein kinase (JNK) activation was closely correlated with the initiation of apoptosis under these conditions. We proposed that a TNF-α-inducible phosphatase was responsible for preventing a sustained activation of JNK and consequent apoptosis in these cells (Guo, Y.-L., Baysal, K., Kang, B., Yang, L.-J., and Williamson, J. R. (1998) J. Biol. Chem. 273, 4027–4034). In the present study we provide further evidence to support this hypothesis. Ro318220, although originally identified as a specific inhibitor of protein kinase C, was subsequently found to be a strong inhibitor of MKP-1 expression. In rat mesangial cells, pretreatment of the cells with Ro318220 blocked expression of MKP-1 induced by TNF-α. This treatment also prolonged JNK activation and caused apoptosis. Taken together, our results support the currently controversial hypothesis that the JNK pathway is involved in TNF-α-induced apoptosis. In addition, we provide a mechanistic explanation for how mesangial cells in primary culture achieve resistance to TNF-α cytotoxicity. Specifically, induction of MKP-1 by TNF-α appears to be responsible for protection of the cells from apoptosis by preventing a prolonged activation of JNK.

Tumor necrosis factor-α (TNF-α) is a polypeptide cytokine that can elicit a wide range of biological responses depending on the cell type and their state of differentiation (1, 2). One of these responses is the induction of apoptosis or programmed cell death in some cell types (3). Although certain tumor cells infected with virus or damaged cells are sensitive to TNF-α-induced apoptosis, many normal cells are usually resistant (3–5). Thus apoptosis has been considered to be an important mechanism for the elimination of abnormal cells and for cellular organization during tissue development.

Most resistant cells can be rendered susceptible to TNF-α-induced apoptosis by agents that block the synthesis of mRNA or protein. Thus, it is proposed that normal cells can achieve resistance to TNF-α cytotoxicity by eliciting the synthesis of a protective factor (6, 7). However, the identities of such protective factors and the mechanisms by which they exert their anti-apoptotic effects are poorly understood. Recent advances in this area have led to some hypotheses. It is proposed that TNF-α activates an anti-apoptotic signaling pathway, such as the extracellular signal-regulated protein kinase (ERK) pathway, which counteracts the cytotoxicity of the apoptotic pathway (8). For example, in L929 cells, fibroblast growth factor-2 suppressed TNF-α-induced apoptosis by activation of ERK, and this effect could be reversed by inhibition of the ERK pathway (9). Another hypothesis favored by recent evidence is that activation of nuclear factor-κB (NF-κB) may be required to protect cells from TNF-α-induced apoptosis in certain cells (6, 7, 10). Increasing evidence indicates that some members of BCL2 family of proteins have inhibitory actions against apoptosis induced by a number of stress signals including TNF-α, probably by blocking caspase activities (3). Given the complexity of TNF-α signaling pathways, it is apparent that different protective factors may exert their anti-apoptotic effects through different mechanisms and act at the different stages of the apoptotic process. The identities and mechanisms of action of the regulatory factors in TNF-α signaling pathways clearly require further investigation under specific cellular conditions.

The role of the JNK pathway has been well documented in various stress-induced models of apoptosis (11–13). However, its involvement in TNF-α-induced apoptosis has been controversial. In a previous report (14), we established a close correlation between the duration of JNK activation and TNF-α-induced apoptosis in rat mesangial cells. We proposed that the JNK pathway is involved in TNF-α-induced apoptosis under conditions that JNK is activated in a sustained manner and that a TNF-α-induced mitogen-activated protein kinase phosphatase-1 (MKP-1) may be responsible for an attenuated JNK effect. This work was supported by National Institutes of Health Grants DK-15120 and DK-48493. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.  

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our hypothesis. In rat mesangial cells, when the expression of MKP-1 induced by TNF-α was selectively blocked by pretreatment of the cells with Ro318220, it produced results that resembled the effects of the protein phosphatase inhibitor vanadate in prolonging JNK activation and inducing apoptosis by TNF-α. Ro318220 selectively blocked expression of MKP-1 without inhibiting the TNF-α stimulated activation of ERK and NF-κB. These results further strengthen our previous conclusion that although TNF-α caused a stimulation of ERK and NF-κB activity, they probably did not contribute to the protective effect against TNF-α-induced apoptosis in mesangial cells. Our studies strongly support the currently controversial hypothesis that the JNK pathway is involved in TNF-α-induced apoptosis. We also provide a novel hypothesis to explain the resistance to TNF-α cytotoxicity in mesangial cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant TNF-α was obtained from Chemicon International Inc. (Temecula, CA). Anti-c-Fos antibodies and anti-MKP-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-c-Jun (Ser 63), anti-c-Jun antibodies were from New England Biolabs (Beverly, MA). Anti-phospho-ERK was from Promega (Madison, WI). Ro318220 was from LC Laboratories (San Diego, CA). The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay kit was from Boehringer Mannheim.

**Cell Culture and Cell Viability Assay**—Rat mesangial cells were isolated from male Sprague-Dawley rats under sterile conditions using the sieving technique as described previously (19). The cells were maintained in RPMI 1640 medium containing 20% fetal calf serum, 0.6 unit/ml of insulin at 37 °C in a humidified incubator (5% CO2, 95% air). Cells from 5–20 passages were used. After the cells were grown to 80–90% confluence, they were made quiescent by incubation for 16–18 h in insulin-free RPMI 1640 medium containing 2% fetal calf serum. For cell viability assays, mesangial cells were grown in 12-well plates. Mesangial cells were treated with various reagents for the indicated times. Uptake of neutral red dye was used as a measurement of cell viability (20). At the end of the incubations, the medium was removed, and the cells were incubated in Dulbecco’s modified Eagle’s medium with 2% fetal calf serum and 0.001% neutral red for 90 min at 37 °C. The uptake of the dye by viable cells was terminated by removal of the medium, washing the cells briefly with 1 ml of 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, and solubilizing the internalized dye by addition of 200 μl of Tris-buffered-saline containing 0.05% Tween 20 and incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies according to the manufacturer’s instructions. The immunoblots were visualized by an ECL kit obtained from Amersham Pharmacia Biotech.

**Immunocytochemical Detection of Apoptosis and c-Jun Phosphorylation**—Cells grown on 25-mm glass coverslips in 6-well plates were fixed in 2% formaldehyde in phosphate-buffered saline, pH 7.4, and solubilized the internalized dye with 0.25% Nonidet P-40. The fixed cells were treated with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) protocol provided by the manufacturer (New England Biolabs). Cell Culture and Cell Viability Assay—The quiescent cells were treated with various reagents as indicated. DNA strand breaks were identified using a TUNEL assay kit (Boehringer Mannheim). Briefly, the fixed cells were treated with terminal deoxynucleotidyl transferase, which incorporates fluorescein tagged nucleotides onto 3’-OH termini of fragmented DNA. Apoptotic nuclei were identified under a fluorescence microscope. Phosphorylation of c-Jun was detected with anti-phospho-c-Jun (Ser 63) antibodies following the immunocytochemistry protocol provided by the manufacturer (New England Biolabs). Positive stained nuclei were visualized with Texas Red-conjugated secondary antibodies using fluorescence microscopy.

**Cell Lysate Preparation**—The quiescent cells were treated with reagents for the indicated times, washed twice with ice-cold phosphate-buffered saline, pH 7.4, and scraped into cell lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM Na2VO4, 50 mM pIophosphate, 100 mM NaF, 1 mM EGTA, 1.5 mM MgCl2, 1% Triton X-100, 100 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μM phenylmethylsulfonyl fluoride. The cells were incubated in lysis buffer for 30 min on ice with periodic vortexing and centrifuged at 15,000 × g for 15 min. The supernatant was designated as the cell lysate. Protein concentration was determined using the method of Bradford using bovine serum albumin as a standard (21).

**Protein Kinase Assays**—JNK activity was measured using a solid phase kinase assay method. GST-c-Jun (1–79) (GST-Jun) fusion protein was isolated from bacterial cells expressing pGEX-c-Jun plasmid. JNK activity was determined using GST-Jun as substrate as described previously (14). Briefly, 100 μg of cell lysate was incubated with 2 μg of GST-Jun agarose beads at 4 °C for 2 h with rotation and centrifuged at 10,000 g for 1 min. The beads were washed three times with washing buffer (25 mM HEPES, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 2.5 mM MgCl2, 0.05% (v/v) Triton X-100, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, and 10 mM NaF). The beads were then resuspended in 10 μl of kinase buffer containing (final concentrations) 20 mM HEPES, pH 7.5, 10 mM MgCl2, 1 mM Na2VO4, 50 mM β-glycerophosphate, 5 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 40 μM ATP, and 1 μCi of [γ-32P]ATP. After incubation at room temperature for 20 min, the reaction was terminated by adding SDS sample buffer followed by heating at 100 °C for 3 min. The proteins were separated on SDS-polyacrylamide gel electrophoresis, and phosphorylated proteins were detected by autoradiography. ERK activation was determined by Western blot analysis using anti-ERK antibodies that only recognize phosphorylated ERK1 and ERK2 (14).

**Western Blot Analysis**—The protein samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered-saline containing 0.05% Tween 20 and incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies according to the manufacturer’s instructions. The immunoblots were visualized by an ECL kit obtained from Amersham Pharmacia Biotech.

**Results**

**Induction of MKP-1 by TNF-α and Effect of Ro318220**—MKP-1 is a well characterized member of MKP family that has been shown to be able to inactivate JNK in vitro and in vivo (16–18). Our previous results showed that MKP-1 mRNA was strongly induced by TNF-α at the same time as JNK inactivation in mesangial cells (14). Beltman et al. (23) reported that Ro318220, which was originally identified as a specific inhibitor of protein kinase C (24), was a strong inhibitor of MKP-1 expression in Rat-1 fibroblasts. To test if it has the same effect on rat mesangial cells, the cells were pretreated with Ro318220 prior to stimulation with TNF-α. As shown in Fig. 1, the expression of MKP-1 induced by TNF-α was totally abolished by pretreatment of the cells with Ro318220, as demonstrated by the Northern blot (Fig. 1A) and the Western blot (Fig. 1B). These results confirm that TNF-α is able to induce de novo synthesis of MKP-1 in rat mesangial cells. The effect of Ro318220 in inhibiting the expression of MKP-1 in mesangial cells is consistent with that observed in Rat-1 cells (23).

**Effects of Ro318220 on Activation of JNK and ERK by TNF-α**—If MKP-1 is responsible for inactivation of JNK as we propose, one would expect that JNK activation induced by TNF-α would be prolonged by blocking the induction of MKP-1. As shown in Fig. 2A, pretreatment of mesangial cells with Ro318220 followed by TNF-α stimulation caused a sustained JNK activation. The effect of Ro318220 on JNK activation is similar to that of the phosphatase inhibitor vanadate as reported previously (14). This result is consistent with the ability of Ro318220 to inhibit the induction of MKP-1 expression induced by TNF-α (Fig. 1) and confirms our prediction that MKP-1 can inactivate JNK in vivo. It is also noted that unlike the situation in Rat-1 cells, where Ro318220 itself is a strong activator of JNK (22), Ro318220 by itself only slightly activated JNK in mesangial cells (Fig. 2A). This response is unlike vanadate, which indiscriminately inhibited all tyrosine phosphatases and caused similar sustained activation patterns for both JNK and ERK in cells pretreated with vanadate followed by TNF-α stimulation (14). Pretreatment of mesangial cells with Ro318220 also potentiated ERK activity, but the major effect was to cause a second activity peak after 2–3 h (Fig. 2B). The reason for the different effects of Ro318220 on JNK and ERK is currently unknown, but it is possible that Ro318220 selectively...
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Results obtained thus far indicate that the JNK and ERK pathways are involved in regulation of the activity and expression of c-Fos and c-Jun (25). To test for a possible downstream effect of MKP-1 on Phosphorylation of c-Jun Stimulated by TNF-α—It is generally recognized that the JNK and ERK pathways are involved in regulation of the activity and expression of c-Fos and c-Jun (25). To test for a possible downstream effect of MKP-1 on c-Jun, the expression of c-Jun and c-Fos was examined (Fig. 3). TNF-α induced the expression of both c-Jun (Fig. 3A) and c-Fos (Fig. 3B) with a stronger effect being observed on c-Fos as judged by Western blot analysis. Pretreatment of the cells with Ro318220 had little effect on TNF-α-induced expression of c-Jun (Fig. 3A), but surprisingly, the TNF-α-induced expression of c-Fos was totally abrogated (Fig. 3B). Ro318220 alone had no apparent stimulatory effect on either c-Jun or c-Fos expression. The most significant effect of Ro318220 on c-Jun was to potentiate its phosphorylation induced by TNF-α (Fig. 3C). This observation is consistent with its ability to sustain JNK activation (Fig. 2A). Although phospho-c-Jun was detectable only at 15 min when cells were stimulated with TNF-α alone, the phosphorylation state of c-Jun lasted for at least 3 h when the cells were treated with a combination of Ro318220 and TNF-α (Fig. 3C). This sustained c-Jun phosphorylation was nearly identical to the pattern caused by pretreatment with vanadate (14).

The phosphorylation of c-Jun was further examined using immunocytochemical analysis in situ. Mesangial cells were pretreated with Ro318220 followed by stimulation with TNF-α. At the end of 3 h of incubation, about 50% of the cells were positively stained with anti-phospho-c-Jun antibodies (Fig. 4D), whereas cells treated with TNF-α alone (Fig. 4B) or Ro318220 alone (Fig. 4C) did not result in detectable phospho-c-Jun stained nuclei compared with control cells, which were not treated with any agent (Fig. 4A).

Effect of TNF-α on the Viability of Mesangial Cells in the Presence or Absence of Ro318220—Results obtained thus far from experiments with Ro318220 on JNK activity and c-Jun phosphorylation stimulated by TNF-α are very similar to those obtained from vanadate experiments as described previously.

Inhibition of MKP-1 Potentiates Apoptosis Induced by TNF-α

Effects of Ro318220 on the expression of c-Jun and c-Fos and on phosphorylation of c-Jun stimulated by TNF-α—Cells were stimulated with 10 ng/ml TNF-α (TNF), treated with 10 μM Ro318220 (Ro), or pretreated with 10 μM Ro318220 for 30 min and then stimulated with 10 ng/ml TNF-α (Ro+TNF) for the times indicated. A, the total mRNA (20 μg) was analyzed by Northern blot using MKP-1 cDNA as a probe. The equal loading of mRNA was judged by 28 S rRNA. B, cells were lysed with SDS sample buffer and heated at 90 °C for 5 min. The samples were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. c-Jun was identified by Western blot analysis using anti-phospho-ERK antibodies. Pretreatment of the cells with Ro318220 had little effect on TNF-α-induced expression of c-Jun (Fig. 3A), but surprisingly, the TNF-α-induced expression of c-Fos was totally abrogated (Fig. 3B). Ro318220 alone had no apparent stimulatory effect on either c-Jun or c-Fos expression. The most significant effect of Ro318220 on c-Jun was to potentiate its phosphorylation induced by TNF-α (Fig. 3C). This observation is consistent with its ability to sustain JNK activation (Fig. 2A). Although phospho-c-Jun was detectable only at 15 min when cells were stimulated with TNF-α alone, the phosphorylation state of c-Jun lasted for at least 3 h when the cells were treated with a combination of Ro318220 and TNF-α (Fig. 3C). This sustained c-Jun phosphorylation was nearly identical to the pattern caused by pretreatment with vanadate (14).

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When the cells were treated with a combination of TNF-α and vanadate, these changes are similar to those produced by Ro318220 (data not shown). These results indicate that TUNEL-stained nuclei showed typical morphological characteristics of apoptosis as examined by light microscopy and further determined by the TUNEL analysis. At the end of 3 h of incubation, about 40% of the cells still attached to the coverslip were TUNEL-stained positive when the cells were treated with a combination of Ro318220 and TNF-α (Fig. 6D), whereas less than 5% of the cells were TUNEL-stained positive after treatment with TNF-α alone (Fig. 6A). This value is close to that in the control experiment (Fig. 6A). Under the same experimental conditions, the number of the nuclei that were shown to be undergoing apoptosis (Fig. 6D) was similar to the number of nuclei stained by phospho-c-Jun antibodies (Fig. 4D). Many TUNEL-stained nuclei and phospho-c-Jun antibody-stained nuclei showed similar characteristics of condensed and irregular apoptotic nuclei, whereas normal nuclei were larger and uniform under phase contrast microscopy (data not shown). These results indicate that TUNEL-stained and phospho-c-Jun antibody-stained cells were the ones undergoing apoptosis. These changes are similar to those produced when the cells were treated with a combination of TNF-α and vanadate (14).

To test if Ro318220 could produce a similar effect on cell viability as that caused by vanadate, the effect of Ro318220 on the viability of mesangial cells in the presence of TNF-α was examined. As shown in Fig. 5, mesangial cells were essentially insensitive to TNF-α cytotoxicity when treated with TNF-α alone. However, when the cells were pretreated with Ro318220, the effect of TNF-α on cell viability was dramatic. Within 4 h of incubation after addition of TNF-α, about 80% of cells were dead, whereas with Ro318220 alone only a slight cellular toxic effect was observed after the same incubation time (Fig. 5). This result is similar to that observed for vanadate potentiation of TNF-α-induced cell death under the same experimental conditions (14). However, unlike vanadate, Ro318220 showed a much less severe cytotoxicity by itself. The effect of Ro318220 to potentiate TNF-α cytotoxicity was dose-dependent; it was evident at a concentration of 2.5 μM and reached a maximum at 15 μM for a 4-h incubation period (data not shown).

Cell death resulting from incubation with Ro318220 and TNF-α showed typical morphological characteristics of apoptosis as examined by light microscopy and further determined by the TUNEL analysis. At the end of 3 h of incubation, about 40% of the cells still attached to the coverslip were TUNEL-stained positive when the cells were treated with a combination of Ro318220 and TNF-α (Fig. 6D), whereas less than 5% of the cells were TUNEL-stained positive after treatment with TNF-α alone (Fig. 6A). This value is close to that in the control experiment (Fig. 6A). Under the same experimental conditions, the number of the nuclei that were shown to be undergoing apoptosis (Fig. 6D) was similar to the number of nuclei stained by phospho-c-Jun antibodies (Fig. 4D). Many TUNEL-stained nuclei and phospho-c-Jun antibody-stained nuclei showed similar characteristics of condensed and irregular apoptotic nuclei, whereas normal nuclei were larger and uniform under phase contrast microscopy (data not shown). These results indicate that TUNEL-stained and phospho-c-Jun antibody-stained cells were the ones undergoing apoptosis. These changes are similar to those produced when the cells were treated with a combination of TNF-α and vanadate (14).

Currently, the mechanism of the potentiation of TNF-α cytotoxicity by Ro318220 is not known. However, as shown in Fig. 5, mesangial cells were essentially insensitive to TNF-α cytotoxicity when treated with TNF-α alone. However, when the cells were pretreated with Ro318220, the effect of TNF-α on cell viability was dramatic. Within 4 h of incubation after addition of TNF-α, about 80% of cells were dead, whereas with Ro318220 alone only a slight cellular toxic effect was observed after the same incubation time (Fig. 5). This result is similar to that observed for vanadate potentiation of TNF-α-induced cell death under the same experimental conditions (14). However, unlike vanadate, Ro318220 showed a much less severe cytotoxicity by itself. The effect of Ro318220 to potentiate TNF-α cytotoxicity was dose-dependent; it was evident at a concentration of 2.5 μM and reached a maximum at 15 μM for a 4-h incubation period (data not shown).

Some inhibitors such as actinomycin D and cycloheximide prior to challenge with TNF-α essentially block all protective factors among various proteins induced by TNF-α (2, 26). It is concluded that protective factor(s) can be elicited by TNF-α to counteract subsequent TNF-α cytotoxicity. However, actinomycin D and cycloheximide essentially block all de novo synthesis of proteins. Therefore, without other approaches, it is not possible to identify specific protective factors among various proteins induced by TNF-α challenge. Molecular genetic techniques have led to identification of some putative protective factors such as NF-κB (6, 10, 27) and BCL2 (28–30). An alternative approach using specific inhibitors to block certain signaling pathways has proved to be useful. For example, PD098059 and pyrrolidine dithiocarbamate have been used to selectively inhibit the ERK pathway and NF-κB activation, respectively (9, 31). The results

**Fig. 4. In situ immunostaining of phospho-c-Jun stimulated by TNF-α and the effect of Ro318220.** A, untreated cells as control. B, cells stimulated with 10 ng/ml TNF-α alone for 3 h. C, cells incubated with Ro318220 for 3.5 h. D, cells pretreated with Ro318220 for 30 min and then stimulated with TNF-α for 3 h. Phospho-c-Jun was detected by anti-phospho-c-Jun antibodies and visualized with Texas Red-conjugated second antibodies by fluorescence microscopy.

**Fig. 5. Effect of TNF-α and Ro318220 on cell viability.** Cells were incubated with 10 ng/ml TNF-α (TNF), 10 μM Ro318220 (Ro), or pretreated with 10 μM Ro318220 for 30 min and then stimulated with 10 ng/ml TNF-α (Ro+TNF) for the times indicated. Cell viability was determined by the neutral red assay method. Results are the means ± S.E. of three experiments performed in triplicate.

**Fig. 6. TNF-α-induced apoptosis of mesangial cells in the presence of Ro318220.** A, untreated cells as control. B, cells stimulated with 10 ng/ml TNF-α alone for 3 h. C, cells incubated with Ro318220 for 3.5 h. D, cells pretreated with Ro318220 for 30 min and then stimulated with TNF-α for 3 h. Apoptotic cell nuclei were identified by TUNEL analysis and visualized by fluorescence microscopy.

**DISCUSSION**

It has been known for many years that most normal cells are resistant to TNF-α cytotoxicity and that this resistance can be abolished if the cells are preincubated with protein synthesis inhibitors such as actinomycin D and cycloheximide prior to exposure to TNF-α. Conversely, preincubation of the sensitive cells with TNF-α increases their resistance to a subsequent challenge with TNF-α (2, 26). It is concluded that protective factor(s) can be elicited by TNF-α to counteract subsequent TNF-α cytotoxicity. However, actinomycin D and cycloheximide essentially block all de novo synthesis of proteins. Therefore, without other approaches, it is not possible to identify specific protective factors among various proteins induced by TNF-α challenge. Molecular genetic techniques have led to identification of some putative protective factors such as NF-κB (6, 10, 27) and BCL2 (28–30). An alternative approach using specific inhibitors to block certain signaling pathways has proved to be useful. For example, PD098059 and pyrrolidine dithiocarbamate have been used to selectively inhibit the ERK pathway and NF-κB activation, respectively (9, 31). The results
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derived from these experiments provided important information for the elucidation of the roles of ERK and NF-κB in the regulation of apoptosis in some cells.

In our previous report, results from experiments with the phosphatase inhibitor vanadate provided us with an important clue that suggested a JNK phosphatase may act as a protective factor against TNF-α toxicity in mesangial cells (14). However, vanadate’s nonspecific inhibition of all protein tyrosine phosphatases, including pre-existing and induced phosphatases, made it difficult to evaluate which enzyme(s) was involved. The fact that MKP-1 mRNA is strongly induced by TNF-α indicates that MKP-1 could be one such phosphatase. If this is the case, one would expect that selectively blocking the expression of MKP-1 would sustain JNK activation and subsequently render the cells susceptible to TNF-α-induced apoptosis. In search of such an approach, it was brought to our attention that Beltman et al. (23) reported recently that Ro318220, originally identified as a PKC inhibitor, selectively inhibited MKP-1 expression induced by epidermal growth factor and PMA in Rat-1 fibroblasts. This observation prompted us to test the effects of Ro318220 on mesangial cells. It showed a similar effect of preventing the expression of MKP-1 induced by TNF-α. More importantly, pretreatment of cells with Ro318220 produced essentially the same effects as those caused by vanadate in sustaining JNK activation, c-Jun phosphorylation, and inducing apoptosis by TNF-α (14). These new results provide substantial evidence to support the notion that MKP-1 can inhibit stimulation of JNK by TNF-α in vitro. To our knowledge, this is the first documentation of MKP-1 induction by TNF-α. MKP are encoded by a multiple gene family. At least eight members have been identified, and virtually all of them are inducible immediate early gene products (15). Whether other members of MKP are inducible by TNF-α and whether they contribute to the resistance of TNF-α apoptotic effect in mesangial cells remains to be investigated. Ro318220 selectively blocked expression of MKP-1 and subsequently prolonged JNK activation without inhibiting the activation of ERK (Fig. 2B) and NF-κB (data not shown) by TNF-α. These results further strengthen our previous conclusion that TNF-α-stimulated ERK and NF-κB activation may not contribute to the protective effect on TNF-α-induced apoptosis (14).

In Rat-1 cells, Ro318220 inhibited PMA- and epidermal growth factor-induced expression of MKP-1 as judged by Western blot analysis. Although it was shown that inhibition of expression of MKP-1 by Ro318220 was not through inhibition of protein synthesis, it was not clear how MKP-1 was inhibited. Here we have demonstrated that Ro318220 blocked MKP-1 expression most likely by inhibiting its transcription in mesangial cells. Ro318220 is a derivative of bisindolylamide and was discovered as a PKC-specific inhibitor (24); however, its effect in blocking the expression of MKP-1 is apparently PKC-independent in Rat-1 cells, suggesting that Ro318220 has some unique properties in addition to being a PKC inhibitor. Our results indicate that the effect of Ro318220 on expression of MKP-1 induced by TNF-α in mesangial cells also seems to be PKC-independent. This is indicated by the fact that blocking the PKC pathway by acute inhibition with another PKC inhibitor, GF109203X, or by down-regulation PKC with PMA only slightly inhibited TNF-α-induced expression of MKP-1. However, both acute inhibition and down-regulation of PKC completely blocked PMA-induced effects under the same conditions. Another interesting observation is that Ro318220 also blocked expression of c-Fos induced by TNF-α in mesangial cells. A similar effect was observed in Rat-1 cells where c-Fos expression induced by lyso phosphatidic acid and PMA was strongly inhibited (23). Further important questions are whether c-Fos induced by TNF-α also plays a role in protecting the cells from apoptosis, and if so, is there any interaction between c-Fos regulated gene products and MKP-1 in the TNF-α signaling pathways. These questions could best be addressed if the expression of c-Fos and MKP-1 induced by TNF-α can be separately manipulated. Experiments attempting to answer these questions are currently under way.

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