Regulation of Inhibitor of Apoptosis Expression by Nitric Oxide and Cytokines: Relation to Apoptosis Induction in Rat Mesangial Cells and RAW 264.7 Macrophages

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Abstract. Mesangial cells and RAW 264.7 macrophages respond to different nitric oxide (NO) donors within 16 to 24 h or 6 to 8 h, respectively, with apoptotic cell death. RAW 264.7 macrophages also die in response to endogenous NO production. In contrast, endogenous NO production fails to significantly induce cell death in mesangial cells. It was hypothesized that differences in the expression of antiapoptotic proteins, in particular the inhibitor of apoptosis (IAP) protein family, might be responsible for this cell type-specific behavior. Therefore, IAP expression was investigated in relation to apoptosis induction of these cell types. In mesangial cells, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) induced cellular inhibitor of apoptosis 1 (cIAP1) mRNA expression within 3 h. In contrast, X chromosome-linked inhibitor of apoptosis (XIAP) mRNA levels remained unaffected by cytokines. Although coinubcation of cells with IL-1β and tumor necrosis factor-α or IL-1β and basic fibroblast growth factor resulted in synergistic induction of inducible NO synthase, comparable potentiating effects on cIAP1 induction were absent. Exogenously released NO from NO donors promoted cIAP1 mRNA upregulation in mesangial cells, whereas XIAP mRNA was downregulated. However, the changes observed on the mRNA level were not adequately translated to the protein level, and corresponding values for cIAP1 and XIAP were only slightly affected. In contrast, in lipopolysaccharide/interferon-γ-stimulated RAW 264.7 macrophages, massive NO-dependent downregulation of cIAP1 and XIAP protein levels, which correlated temporally with the induction of apoptosis, was observed. This effect was at least partially reversed by Nω-monomethyl-L-arginine, an inhibitor of NO synthase activity. In summary, a direct correlation between the downregulation of IAP protein levels and the induction of apoptosis by endogenous NO was observed in macrophages. In contrast, a stable level of IAP protein in mesangial cells might represent a mechanism for the resistance of the cells to endogenously produced NO.

Glomerular inflammatory diseases are a leading cause of end-stage renal failure. Most forms of human glomerulonephritis lack specific therapy, and the mechanisms involved in the initial injury to the kidney and the progression to renal scarring (glomerulosclerosis and interstitial fibrosis) are still not completely understood (1). Some forms of human glomerulonephritis may resolve, allowing glomerular structure and function to return to normal. In progressive forms of glomerulonephritis, glomerular inflammation is sustained and eventually leads to end-stage renal failure. The glomerulus is a well developed capillary network, and mesangial cells appear to play a pivotal role in glomerulonephritis and several other pathologic conditions (2). Cellular proliferation of mesangial cells or their removal by apoptosis is thought to be pivotal for the development of mesangial cell proliferation and the progression of several forms of glomerulonephritis. After cell activation, as a result of cytokine release by mesangial cells themselves or by infiltrating macrophages, mesangial cells respond with proinflammatory mediator secretion and inducible nitric oxide (NO) synthase (iNOS) induction (3).

NO is a highly versatile and short-lived messenger that mediates a number of diverse physiologic and pathophysiologic processes. One of the most intensively investigated features of NO signaling is its effect on cell survival and cell death (4). Several years ago, NO derived from an iNOS isoform was initially reported to induce apoptotic cell death in macrophages; the involvement of NO was demonstrated with the use of specific NO synthase inhibitors (5). Apoptosis is distinguishable from necrosis by specific biochemical and structural events, such as DNA ladder formation, chromatin condensation, and finally fragmentation into apoptotic bodies. NO has emerged as a bifunctional regulator of apoptosis (6), whose function depends on the cell type and the amount of NO released within a specific time period. Large amounts of NO released exogenously by NO donors or endogenously via iNOS induction result in apoptotic alterations in, for example, RAW 264.7 macrophages (7). Apoptotic death signaling involves, at least in part, accumulation of the tumor suppressor p53 (8,9), activation of caspases (10,11), and altered expression of Bcl-2.
family proteins (12). Moreover, copper/zinc superoxide dismutase protein and mRNA downregulation (13) and stress-activated protein kinase activation (14) have been described. In addition to RAW 264.7 macrophages, which have been extensively characterized in terms of NO-induced apoptotic signal transduction, numerous studies have confirmed the ability of NO to initiate apoptosis in several other cell types, including pancreatic β-cells, neuronal cells, mast cells, chondrocytes, thymocytes, and renal mesangial cells (15). In contrast, many reports indicated that NO or related molecules of endogenous or exogenous origin could inhibit programmed cell death in a variety of cells and tissues (16). Initially, protective effects of NO were observed in Epstein-Barr virus-infected B lymphoma cell lines that constitutively expressed iNOS and exhibited an increased spontaneous apoptosis rate when exposed to N\textsubscript{G}\textsuperscript{-}\textit{)-monomethyl}-l-arginine (l-NMMA) (17). Antiapoptotic effects of NO were subsequently demonstrated in several other cell types, such as hepatocytes, eosinophils, endothelial cells, thymocytes, and embryonic kidney cells (16). The general protective function of NO seems to be largely independent of the apoptotic stimulus (4). As one potential protective mechanism, NO-dependent S-nitrosylation of several caspase isoenzymes, such as caspases 3 and 8, and concomitant inhibition of caspase enzyme activity were demonstrated mainly in in vitro studies (18).

One family of proteins that may extensively modulate apoptosis signal transduction is the inhibitor of apoptosis (IAP) family of proteins (19). IAP were first identified in insect viruses and seem to be evolutionarily conserved proteins that are characterized by baculovirus inhibitor repeat (BIR) domains and in part by a carboxyl-terminal, zinc-binding, RING motif (20). In human cells, several IAP relatives have been identified, i.e., cellular inhibitor of apoptosis 1 (cIAP1), cIAP2, X chromosome-linked inhibitor of apoptosis (XIAP), neuronal apoptosis inhibitory protein, survivin, and apollon. Mouse orthologs of most human IAP have also been identified, whereas IAP family members in rat cells are only poorly characterized [cIAP1 correlates with rat inhibitor of apoptosis 1 (RIAP1), cIAP2 correlates with RIAP2, and XIAP correlates with RIAP3]. Functionally, cIAP1, cIAP2, XIAP, and survivin were demonstrated to bind to and potently inhibit caspases 3, 7, and 9 but not caspases 1, 6, 8, or 10 (21,22). In addition to directly affecting effector proteases, the IAP family of proteins was linked to signal transduction pathways used by members of the tumor necrosis factor (TNF) receptor family (19). cIAP1 and cIAP2 are recruited to the TNF receptor by binding to TNF receptor-associated factor-1/TNF receptor-associated factor-2 heterocomplexes (23), which seem to be functionally implicated in TNF induction of nuclear factor κB (NF-κB) and protection from apoptosis (24).

In this work, we focused on the role of IAP family proteins during NO-induced apoptosis in rat mesangial cells and mouse RAW 264.7 macrophages. RAW 264.7 cells die by apoptosis in response to exogenously or endogenously released NO, and we observed a massive downregulation of cIAP1 and XIAP protein levels. In contrast, in rat mesangial cells, which are resistant to endogenously released NO and rarely die by apoptosis in response to NO donors (25), cIAP1 (RIAP1) and XIAP (RIAP3) proteins remained constitutively expressed. Therefore, we suggest that NO-mediated IAP regulation constitutes an additional mechanism for the positive or negative regulation of apoptosis by NO.

**Materials and Methods**

**Materials**

RNase A, RNase T1, and RNA polymerases T3 and T7 were from Roche Molecular Biochemicals (Mannheim, Germany). Diphenylnamine, lipopolysaccharide (LPS) (Escherichia coli serotype 0127: B8), forskolin, and guanidine isothiocyanate were purchased from Sigma (Deisenhofen, Germany). Spermine/NO, diethylenetriamine (DETA)/NO, and l-NMMA were supplied by Alexis (Freiburg, Germany). Human basic fibroblast growth factor (bFGF) was purchased from R&D Systems (Wiesbaden, Germany), human interleukin-1β (IL-1β) was from Cell Concepts (Umkirch, Germany), and recombinant human TNF-α (specific activity, 6.6 × 10\textsuperscript{6} U/mg) was a generous gift from Knoll AG (Ludwigshafen, Germany). RPMI 1640 medium, cell culture supplements, and fetal calf serum were from Life Technologies (Egggenstein, Germany). All other chemicals were of the highest grade of purity commercially available.

**Cell Culture and Cell Treatment**

Rat renal mesangial cells were cultured and characterized as described previously (26). In a second step, single cells were cloned by limiting dilution in 96-well plates. Clones with apparent mesangial cell morphologic features were characterized by positive staining for the intermediate filaments desmin and vimentin (which are considered to be specific for myogenic cells), positive staining for Thy.1.1 antigen, and negative staining for factor VIII-related antigen and cyto-keratin (to exclude endothelial and epithelial contaminations, respectively). For the experiments in this study, passages 13 to 23 were used.

Cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. For all experiments, subconfluent cells were starved for 24 h and exposed to the different substances in starvation medium, i.e., Dulbecco’s modified Eagle’s medium containing 0.1 mg/ml fatty acid-free bovine serum albumin, 100 U/ml penicillin, and 100 µg/ml streptomycin.

**S-Nitrosoglutathione Synthesis**

S-Nitrosoglutathione (GSNO) was synthesized as described previously (8,27). Briefly, glutathione was dissolved in HCl at 4°C before the addition of NaNO\textsubscript{2}. The mixture was stirred at 4°C for 40 min, followed by the addition of 2.5 vol of acetone. Precipitates were filtered, washed with acetone and diethyl ether, and dried under vacuum. GSNO was characterized by HPLC analysis and ultraviolet spectroscopy.

**Quantitation of DNA Fragmentation**

DNA fragmentation was assayed essentially as reported previously (12). Briefly, after incubation, cells were scraped off the culture plates, resuspended in 250 µl of 10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA) (Tris-EDTA buffer, pH 8.0), and incubated for 30 min at 4°C with an additional 1 vol of lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100). After lysis, intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at 13,000 × g. Pellets were resuspended in 500 µl of Tris-EDTA buffer, and samples were precipitated by addition of 500 µl of 10% TCA at 4°C. Samples were pelleted at 4000
Biochemicals. The protocol described by the manufacturers (Roche Molecular Biochemicals).

**Probe DNA**

All cDNA fragments used as probes for RNase protection assays were cloned by PCR, using the following nucleotide primers (Life Technologies). For the rat IAP1 probe, 5'-CTCATG(CG)ACAAACTG(cT)CTCC-3' was used as the 5'-primer and 5'-CT(AG)GG(TA)GT(CT)AG(CT)(TG)TGCC-3' was used as the 3'-primer. For the rat XIAP (RIAP3) probe, 5'-TGACTTTT TAACGATTTTGAAGG-3' was used as the 5'-primer and 5'-GTCTCTCACAAC(TA)(TC)TGACTGCTC-3' was used as the 3'-primer. For the rat XIAP (RIAP3) probe, 5'-TGACTTTT TAACGATTTTGAAGG-3' was used as the 5'-primer and 5'-GTCTCTCACAAC(TA)(TC)TGACTGCTC-3' was used as the 3'-primer. For the rat XIAP (RIAP3) probe, 5'-TGACTTTT TAACGATTTTGAAGG-3' was used as the 5'-primer and 5'-GTCTCTCACAAC(TA)(TC)TGACTGCTC-3' was used as the 3'-primer. For the rat XIAP (RIAP3) probe, 5'-TGACTTTT TAACGATTTTGAAGG-3' was used as the 5'-primer and 5'-GTCTCTCACAAC(TA)(TC)TGACTGCTC-3' was used as the 3'-primer. For the rat XIAP (RIAP3) probe, 5'-TGACTTTT TAACGATTTTGAAGG-3' was used as the 5'-primer and 5'-GTCTCTCACAAC(TA)(TC)TGACTGCTC-3' was used as the 3'-primer. For the rat XIAP (RIAP3) probe, 5'-TGACTTTT TAACGATTTTGAAGG-3' was used as the 5'-primer and 5'-GTCTCTCACAAC(TA)(TC)TGACTGCTC-3' was used as the 3'-primer. For the rat XIAP (RIAP3) probe, 5'-TGACTTTT TAACGATTTTGAAGG-3' was used as the 5'-primer and 5'-GTCTCTCACAAC(TA)(TC)TGACTGCTC-3' was used as the 3'-primer. For the rat XIAP (RIAP3) probe, 5'-TGACTTTT TAACGATTTTGAAGG-3' was used as the 5'-primer and 5'-GTCTCTCACAAC(TA)(TC)TGACTGCTC-3' was used as the 3'-primer.

**RNA Isolation and RNase Protection Analyses**

RNA isolation was performed as described (29). Twenty micrograms of total RNA for the different experimental time points of the cell culture experiments were used for RNase protection assays.

DNA probes were cloned into the transcription vector pBluescript II KS(+) (Stratagene, Heidelberg, Germany) or pCR2.1-TOPO (Invitrogen, Leek, Netherlands) and linearized. An antisense transcript was synthesized in vitro by using RNA polymerase T3 or T7 and [γ-32P]UTP (800 Ci/mmol; Amersham, Freiburg, Germany). RNA samples were hybridized overnight at 42°C with 100,000 cpm of the labeled antisense transcript. Hybrids were digested with RNase A and T1 for 1 h at 30°C. Under these conditions, every single mismatch is recognized by the RNases. Protected fragments were separated on 5% acrylamide/8 M urea gels and analyzed using a PhosphorImager (Raytest, Straubenhardt, Germany). All protection assays were performed with at least three different sets of RNA from independent experiments.

**Western Blot Analyses**

Cells were cultured and incubated as described. Cell lysis was achieved with lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, pH 8.0) and sonication (Branson sonifier; duration, 10 s; duty cycle, 100%; output control, 10%), followed by centrifugation (4000 × g, 5 min) and protein determination using the method described by Bradford (30). Proteins were normalized to 50 µg/lane or 10 µg/lane (RIAP3), resolved on 7.5% (cIAP1/RIAP1) or 10% (XIAP/RIAP3) polyacrylamide gels, and blotted onto polyvinylidene difluoride sheets. Sheets were washed twice with Tris-buffered saline (TBS) (140 mM NaCl, 50 mM Tris, pH 7.2) containing 0.1% Tween-20 before nonspecific binding was blocked with TBS with 5% skim milk. Filters were incubated overnight at 4°C with either mouse anti-human/mouse human IAP-like protein antibody (clone 28, 1:250 in TBS with 0.5% skim milk; Transduction Laboratories) to assay mouse XIAP expression, mouse anti-human/mouse human IAP-like protein antibody (clone 48, 1:12,500 in TBS with 0.5% skim milk; Transduction Laboratories) to assay RIAP3 expression, or rabbit anti-cIAP1 antibody (1 µg/ml in TBS with 0.5% skim milk; Santa Cruz Biochemicals, Santa Cruz, CA) to assay mouse cIAP1 and RIAP1 expression. Sheets were washed five times, and nonspecific binding was blocked as described. Detection was performed for 1.5 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse monoclonal antibodies (1:5000) or goat anti-rabbit monoclonal antibodies (1: 5000), using the enhanced chemiluminescence method (Amersham).

**Metabolic Labeling**

For the detection of new RIAP1 protein synthesis, confluent rat mesangial cells were starved for 20 h with methionine-free minimal essential medium containing 0.1 mg/ml bovine serum albumin and the appropriate stimuli. Sixteen hours after starvation, 0.14 µCi of [35S]methionine (Amersham) was added to each plate for an additional period of 4 h. After incubation and labeling, cells were lysed in 1 ml of lysis buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 2 mM ethylene glycol bis(b-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA), 40 mM β-glycero-phosphate, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM phenyl-methylsulfonyl fluoride, 100 mM okadaic acid, 200 µM sodium orthovanadate, 2 mM diithiothreitol] and homogenized with 10 strokes of a 26-gauge needle. After centrifugation (13,000 × g, 10 min, RIAP1 was immunoprecipitated overnight at 4°C in lysis buffer containing 5% fetal calf serum, 0.5 mM iodoacetamide, 1 µg/ml anti-RIAP1 antibody (Santa Cruz), and protein A/G-Sepharose. Beads were washed twice with low-salt buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.1% sodium dodecyl sulfate (SDS)] and three times with high-salt buffer (50 mM Tris, pH 7.4, 500 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.1% SDS) before a 5-min heating period at 95°C in 50 µl of LD buffer (100 mM Tris, pH 7.4, 10% glycerol, 5% SDS, 10% β-mercaptoethanol). After gel electrophoresis, labeled proteins were detected by PhosphorImager analysis.

**Statistical Analyses**

Each experiment was performed at least three times. Statistical analyses were performed using the two-tailed t test or ANOVA. For multiple comparisons, the data were corrected using Dunn’s method.

**Results**

**Effects of NO on RAW 264.7 and Mesangial Cell Viability**

In several previous studies, we and others documented that NO donors potently induced apoptotic cell death in mouse RAW 264.7 macrophages within 8 h (1 mM GSNO, approxi-
mately 35% DNA fragmentation; 250 μM spermine/NO, approximately 40% DNA fragmentation) (7,8,31). Also, NO released endogenously via iNOS induction elicited by LPS/interferon-γ (IFN-γ) caused apoptotic cell death in approximately 40 to 50% of the stimulated cells within 24 h (10). Similar to findings for RAW 264.7 macrophages but with different kinetics, NO donors such as spermine/NO, GSNO, S-nitroso-N-acetylpenicillamine, and a NO-saturated solution caused apoptosis in rat mesangial cells (25). We confirmed these results and demonstrated apoptotic DNA fragmentation within 24 h, which amounted to approximately 22% with 375 μM spermine/NO, approximately 22% with 750 μM GSNO, and approximately 15% with 1 mM DETA/NO (Figure 1). Apoptosis was confirmed by morphologic investigations using Hoechst dye 33258 and fluorescence microscopy, which demonstrated that all NO-releasing compounds induced chromatin condensation and all other signs of nuclear pyknosis (25).

Next, we investigated whether endogenously released NO also affects mesangial cell viability. IL-1β elicited iNOS induction and long-lasting release of NO but only weakly elicited apoptotic cell death (Figure 2). Only iNOS superinduction by IL-1β and bFGF significantly potentiated NO release and led to approximately 9% apoptotic DNA fragmentation (Figure 2A) (32) and, correspondingly, approximately 8 to 9% apoptotic cells (data not shown). The data obtained with the quantitative diphenylamine assay were qualitatively confirmed with a DNA/histone-based enzyme-linked immunosorbent assay (Figure 2B) and with morphologic investigations using Hoechst dye 33258 staining (data not shown).

Effects of Cytokines and NO on RIAP1 and RIAP3 Expression in Mesangial Cells

Gene expression of IAP, which are essential regulators of apoptosis signal transduction (19), is known to be affected by TNF-α via NF-κB activation (33). Therefore, we first investigated whether there was a direct correlation between resistance against NO-induced apoptosis and IAP gene expression in rat mesangial cells. As shown in Figure 3B, TNF-α potently and rapidly (within 3 h after TNF-α stimulation) elicited RIAP1 gene expression; elevated RIAP1 expression was detectable for
>24 h (control levels of RIAP1 mRNA were sustained at the 3-h level during the entire incubation period; data not shown). Like TNF-α, IL-1β was able to induce RIAP1 gene expression, beginning 3 h after treatment, and continuously increasing mRNA induction was detectable up to 24 h (Figure 3A). We next investigated whether bFGF, which synergistically potentiated IL-1β-induced iNOS expression, also affected IL-1β-induced RIAP1 gene expression. As demonstrated in Figure 3A, bFGF had no effect alone on RIAP1 expression and was not able to modulate IL-1β-mediated RIAP1 expression. Also, in contrast to iNOS expression, the combination of IL-1β and TNF-α had no potentiating effect on RIAP1 gene expression (Figure 3B). None of the tested cytokines affected RIAP3 mRNA expression (Figure 3).

In the following set of experiments, we examined a potential effect of endogenously released NO on IL-1β- or IL-1β/bFGF-mediated RIAP1/RIAP3 gene expression. For these experiments, we coincubated rat mesangial cells with the appropriate cytokines in the absence or presence of 2 mM L-NMMA, which potently blocked endogenous NO release (34). As shown in Figure 4, RIAP1 mRNA induction by IL-1β or IL-1β/bFGF was not affected by the presence of L-NMMA during the first 8 h. In contrast, the maximal response obtained after 24 h of cytokine stimulation was significantly lower in the presence of L-NMMA. With the delayed induction of iNOS in mesangial cells (34), this clearly demonstrates a contribution of endogenous NO to RIAP1 mRNA expression in mesangial cells. As observed

Figure 3. Effects of cytokines on rat inhibitor of apoptosis 1 (RIAP1) and RIAP3 mRNA expression in glomerular mesangial cells. Cells were rendered quiescent by serum starvation for 24 h; cells were then stimulated for different time periods with 2 nM IL-1β and/or 10 ng/ml bFGF or left untreated (control) (A) or were stimulated with 30 ng/ml TNF-α and/or 2 nM IL-1β (B). Samples of 20 μg of total RNA were analyzed for RIAP1 and RIAP3 mRNA expression in RNase protection assays. In each experiment, 1000 counts/min of the hybridization probe were used as size markers. Representative RNase protection gels for each gene (RIAP1 and RIAP3) are presented in the upper panels. Control values remained at the same level throughout the incubation period (data not shown). The same sets of total RNA were also analyzed for expression of β-actin, as a loading control. The RIAP1 and RIAP3 mRNA expression levels were assessed by PhosphorImager analysis of the radiolabeled gels, and PhosphorImager data are presented graphically in the lower panels. Data are means ± SEM of three or four separate experiments.
with exogenous NO, RIAP3 expression was not altered by endogenous NO (Figure 4).

To confirm a direct modulatory effect of NO on RIAP1 gene expression, we exposed mesangial cells to spermine/NO and DETA/NO. Both NO donors rapidly elicited RIAP1 gene upregulation, within 3 h (Figure 5). However, continuous exposure of cells to NO donors for up to 24 h revealed that RIAP1 gene upregulation by NO was only transient. This was true for spermine/NO as well as for DETA/NO. In contrast to RIAP1, RIAP3 mRNA expression was downregulated by both NO donors, beginning 8 h after NO donor addition. Interestingly, costimulation by NO donors and TNF-α led to superinduction of RIAP1 and enhanced RIAP3 downregulation. Therefore, the exogenously added NO may compensate for the poor NO-inducing capability of TNF-α and may result in a synergistic effect on RIAP1 expression comparable to that observed with IL-1β, which potently triggered endogenous NO release (Figure 4).

In the next step, we investigated whether cytokine- or NO-induced RIAP1 mRNA expression was translated into changes in RIAP1 protein expression. As documented in Figure 6A, TNF-α caused a slight but significant increase in RIAP1 protein expression within 24 h. In contrast, IL-1β and IL-1β/bFGF only weakly affected RIAP1 protein expression. Similarly, NO

![Figure 4](https://example.com/image4.png)
donors such as spermine/NO and DETA/NO only marginally affected RIAP1 protein levels (Figure 6A). We detected no significant change in RIAP3 protein expression within 24 h with either cytokines or NO donors, although RIAP3 mRNA was downregulated by NO (Figure 6B).

To mechanistically address the apparently discrepant behavior of the RIAP1 protein and mRNA levels, we used the translational inhibitor cycloheximide and performed metabolic labeling experiments. Incubation of rat mesangial cells with [35S]methionine revealed an approximately fivefold increase in de novo synthesis of RIAP1 after TNF-α stimulation (Figure 7A). This increased RIAP1 biosynthesis, which probably reflects the increased RIAP1 mRNA levels, must be counterbalanced by a concomitant increase in RIAP1 protein degradation to explain the unaltered steady-state levels of RIAP1 protein. Experiments using cycloheximide revealed that RIAP1 was degraded by approximately 30%. Coincubation of cells with cycloheximide and TNF-α moderately enhanced RIAP1 degradation (Figure 7B). These experiments indeed indicate the regulation of RIAP1 protein at the levels of both protein synthesis and protein degradation.

Figure 5. Upregulation of RIAP1 but downregulation of RIAP3 mRNA levels in rat mesangial cells by NO donors. Cells were rendered quiescent by serum starvation for 24 h, followed by stimulation for different time periods with 30 ng/ml TNF-α, 250 μM spermine/NO, 250 μM spermine, or 30 ng/ml TNF-α plus 250 μM spermine/NO (A) or 30 ng/ml TNF-α, 1 mM DETA/NO, or 30 ng/ml TNF-α plus 1 mM DETA/NO (B). Samples of 20 μg of total RNA were analyzed for RIAP1 and RIAP3 mRNA expression in RNase protection assays. In each experiment, 1000 counts/min of the hybridization probe were used as size markers. Representative RNase protection gels for each gene (RIAP1 and RIAP3) are presented in the upper panels. Control values remained at the same level throughout the incubation period (data not shown). The same sets of total RNA were also analyzed for expression of β-actin, as a loading control. The RIAP1 and RIAP3 mRNA expression levels were assessed by PhosphorImager analysis of the radiolabeled gels, and PhosphorImager data are presented graphically in the lower panels. Data are means ± SEM of three or four separate experiments.
Effects of NO on cIAP1 and XIAP Expression in Mouse RAW 264.7 Macrophages

Because the effect of NO on RIAP1 expression in mesangial cells was limited to mRNA regulation, with only marginal changes in RIAP1 and RIAP3 protein levels, we next investigated the effects of NO on cIAP1 and XIAP mRNA and protein levels in mouse RAW 264.7 macrophages. In contrast to mesangial cells, RAW 264.7 macrophages rapidly die after exposure to NO, either exogenously released or endogenously produced in response to LPS/IFN-γ stimulation. As in mesangial cells, NO donors such as GSNO and spermine/NO produced an increase in cIAP1 mRNA levels within 3 to 8 h (Figure 8, A and B). In contrast, XIAP mRNA levels remained unaffected. Figure 8, C and D, demonstrates that LPS/IFN-γ temporally upregulate cIAP1 mRNA within 2 to 4 h, followed by a decrease back to control levels at 18 h and further downregulation at 24 h. Surprisingly, blockade of NO release with the application of 1 mM l-NMMA left cIAP1 mRNA regulation unaffected except at the 24-h time point, when l-NMMA blocked cIAP1 mRNA downregulation. This indi-

Figure 6. Effects of cytokines and NO on RIAP1 and RIAP3 protein expression in rat mesangial cells. Cells were rendered quiescent by serum starvation for 24 h, followed by stimulation for different time periods with 2 nM IL-1β, 10 ng/ml bFGF, 2 nM IL-1β plus 10 ng/ml bFGF, 30 ng/ml TNF-α, 2 nM IL-1β plus 30 ng/ml TNF-α, 1 mM DETA/NO, 1 mM DETA/NO plus 30 ng/ml TNF-α, 250 μM spermine/NO (Sp/NO), 250 μM spermine/NO plus 30 ng/ml TNF-α, or vehicle (control), as indicated. Proteins were subsequently normalized to 50 μg/lane, and cell lysates were subjected to Western blot analysis for RIAP1 [cellular inhibitor of apoptosis 1 (cIAP1)] (A) and RIAP3 [X chromosome-linked inhibitor of apoptosis (XIAP)] (B) proteins, followed by enhanced chemiluminescence detection and video densitometry. Each blot is representative of three similar experiments.
cates that NO, via eventual activation of proapoptotic pathways, led to cIAP1 mRNA downregulation concomitantly with apoptosis induction. Moreover, LPS/IFN-γ elicited rapid and NO-independent downregulation of XIAP mRNA levels within 8 to 24 h (Figure 8, C and D).

Next, cIAP1 and XIAP protein expression was investigated by Western blot analysis. As demonstrated in Figure 9, GSNO and spermine/NO potently induced cIAP1 protein downregulation beginning 2 to 3 h after addition of the NO donor. cIAP1 protein downregulation/degradation temporally preceded the first signs of apoptosis induction, such as DNA laddering and apoptotic morphologic features (which first occur approximately 4 to 5 h after NO donor addition) (7), and appeared together with p53 upregulation (10). In contrast, XIAP protein levels were not altered by NO donor exposure. Similarly to cIAP1 mRNA regulation, cytokines elicited a biphasic effect: 3 to 6 h after LPS/IFN-γ addition; cIAP1 protein expression was downregulated, followed by upregulation at 10 to 14 h and finally degradation at 24 h. Coexposure to L-NMMA demonstrated that the final downregulation event at 24 h depended on NO release. Similarly, XIAP protein levels were downregulated by LPS/IFN-γ within 18 to 24 h, which correlates with the time course of apoptosis induction. Again, 1 mM L-NMMA blocked LPS/IFN-γ-induced XIAP protein downregulation (Figure 9).

**Discussion**

**NO and Its Effects on Cell Viability**

For several years, NO was described as an inducer of apoptotic cell death in many different cell systems. Extensive studies using NO donors in RAW 264.7 macrophages, rat mesangial cells, chondrocytes, pancreatic β-cells, neuronal cells, and other cell types were undertaken (15). Moreover, endogenous release of NO via iNOS induction was reported to elicit apoptotic cell death, particularly in RAW 264.7 macrophages and neuronal cells (7). However, some cell types seem to be resistant to NO-induced cell death, such as endothelial cells and glomerular epithelial cells (Meßmer UK, Pfeilschifter J, unpublished observations), or to discriminate between exogenous and endogenous NO release (25). We clearly demonstrated that rat mesangial cells produced large amounts of NO in response to stimulation with IL-1β plus bFGF or IL-1β plus TNF-α; the amounts of NO were comparable to those produced by RAW 264.7 macrophages stimulated with LPS/IFN-γ. Whereas RAW 264.7 macrophages die NO-dependently within 24 h, rat mesangial cells were highly resistant to endogenously produced NO (Figure 2) (25). Moreover, RAW 264.7 macrophages exhibited homogeneous iNOS expression and apoptotic nuclei in the iNOS-containing cells after stimulation with IFN-γ and LPS, whereas mesangial cells that expressed iNOS did not display signs of apoptosis and, conversely, cells that exhibited characteristic features of apoptosis did not stain for iNOS (35). One possible explanation is that IL-1β not only induces the expression of iNOS but also stimulates a protecting mediator in mesangial cells. In line with this hypothesis are experiments demonstrating that preincubation of mesangial cells with IL-1β attenuated DNA damage attributable to exogenous NO by approximately 50% (35). One such protective factor may be a member of the Bcl-2 family of proteins, such as Bcl-2 itself, which is upregulated in response to simultaneous generation of NO and O₂⁻ (36). Similar to findings with balanced cogeneration of NO and O₂⁻, preactivation of RAW 264.7 macrophages with nontoxic concentrations of NO donors or the redox cycler 2,3-dimethoxy-1,4-naphthoquinone for 15 h attenuated GSNO-initiated apoptotic cell death and averted accumulation of p53, which is indicative of macrophage apoptosis (37). Mechanistically, the promotion of cyclooxygenase-2 expression by NF-κB and activator protein-1 activation seems to mediate protection against NO-induced cytotoxicity (38). Other known mechanisms that have been suggested to provide resistance to NO include the activation of MEK kinases, because the MEK inhibitor PD98059 reverses resistance (39), and the upregulation of superoxide dismutase expression (13). Moreover, nontoxic concentrations of NO were reported to block the activity of certain caspases, such as caspase 3, via
S-nitrosylation (18,40). However, the in vivo relevance of the latter observation remains to be proven. In this work, we describe another family of proteins that are regulated by NO and may be involved in resistance to NO, namely the IAP family of proteins.

Evidence that NO Is a Direct Inducer of cIAP1 mRNA

At least five different mammalian IAP family members (XIAP, cIAP1, cIAP2, neuronal apoptosis inhibitory protein, and survivin) exhibit antiapoptotic activity in cell culture. IAP are able to block a broad spectrum of apoptotic stimuli, including members of the TNF receptor family, proapoptotic members of the Bcl-2 family, cytochrome c, and chemotherapeutic agents. XIAP seems to have the broadest and strongest antiapoptotic activity (41). For at least some IAP, the antiapoptotic potential can be explained by the inhibition of certain caspases. The IAP/caspase ratio might be modulated by transcriptional or translational regulation of both groups of proteins, or IAP might be regulated by localization, post-translational modifications, binding by other proteins, or degradation (42). Here

Figure 8. NO mediation of cIAP1 mRNA upregulation concomitant with apoptotic cell death induction in mouse RAW 264.7 cells. Cells were treated for the times indicated with 1 mM GSNO (A), 350 μM spermine/NO (B), 10 μg/ml lipopolysaccharide (LPS) plus 100 U/ml interferon-γ (IFN-γ) (C), 10 μg/ml LPS plus 100 U/ml IFN-γ plus 1 mM l-NMMA (D), or vehicle (control). Samples of 20 μg of total RNA were analyzed for RIAP1 and RIAP3 mRNA expression in RNase protection assays. In each experiment, 1000 counts/min of the hybridization probe were used as size markers. Representative RNase protection gels for each gene (RIAP1 and RIAP3), from three independent experiments, are presented. The same sets of total RNA were also analyzed for expression of β-actin, as a loading control.
we provide evidence that NO acts as a potent transcriptional regulator of cIAP1, cIAP2, and XIAP in human endothelial cells by IL-1β, TNF-α, or LPS (33) and marked induction of the IAP family proteins survivin and XIAP by vascular endothelial growth factor (43). Concerning signaling pathways leading to IAP upregulation, several studies demonstrated a dependence on NF-κB transcription factor activity by using transient or stable transfection with inhibitor κBα (33,44–46). Because NO and NO-releasing compounds have been reported to activate NF-κB (47), it can speculated that NO-induced IAP mRNA induction is mediated by NF-κB activation. However, the effect of NO on NF-κB activation is controversial. NF-κB activation by NO was reported for lymphocytes (48) and for liver and lung during hemorrhagic shock (49); however, NO inhibits NF-κB activation in part by hindering DNA binding at least in vitro (50). Similar to NO modulation of cell death, the NF-κB-activating activities may be attributable to small concentrations of NO and the NF-κB-blocking effects may result from high concentrations of NO. However, because toxic NO donor concentrations also result in cIAP1 mRNA upregulation, alternative mechanisms, such as activator protein-1 activation by NO (47,51), must be considered. Further work is needed to investigate this matter.

**NO, Cell Death, and IAP Protein Levels**

One of our major findings is that mesangial cells, which are highly resistant to endogenously released NO and die with slow kinetics in response to high doses of NO donors, maintain cIAP1 and XIAP protein expression at high levels, whereas RAW 264.7 macrophages, which rapidly die in response to NO, demonstrate massive rapid cIAP1 and XIAP downregulation in response to LPS/IFN-γ. Consistently, there was a direct correlation between cIAP1 downregulation and rapid induction of apoptotic cell death by NO.

Surprisingly, in our experiments cIAP1 mRNA upregulation by cytokines or NO did not result in cIAP1 protein upregulation in mesangial cells, indicating that changes in IAP mRNA levels did not necessarily translate into changes in protein levels. In vivo labeling experiments and protein stability studies using cycloheximide revealed an increase in new IAP1 protein biosynthesis that was accompanied by an increase in protein degradation, thus explaining the constant IAP1 protein levels in rat mesangial cells after cytokine or NO stimulation. Similar findings were recently reported for different tumor cell lines, in which XIAP, cIAP1, and cIAP2 mRNA levels did not correlate with protein levels, indicating post-transcriptional regulation of expression (52). Post-transcriptional regulation of IAP protein levels was previously described for cIAP1 and XIAP, levels of which were substantially decreased during thymocyte apoptosis elicited by dexamethasone (53), and for XIAP, which is cleaved into two fragments during Fas-induced apoptosis (42). In the former report, IAP were degraded in a proteasome-dependent manner before cell death, which indicates that IAP degradation is a prerequisite for thymocyte apoptosis; in the latter report, XIAP was cleaved into two fragments, comprising either the BIR-1 and -2 domains or the BIR-3 domain and the RING domain and
exhibiting abilities to block caspase 3 and 7 or caspase 9, respectively. We now report on cIAP1 downregulation during NO-induced apoptosis of RAW 264.7 macrophages. It is tempting to speculate that, as in thymocytes, efficient NO-induced apoptosis requires IAP downregulation/degradation. Differences in cIAP1 protein levels between rat mesangial cells and RAW 264.7 macrophages, resulting from differences in specific degradation pathways, may explain the different sensitivities to NO-induced programmed cell death. For mechanistic evaluation of the role of IAP downregulation in RAW 264.7 macrophages, IAP overexpression and IAP antisense experiments will be required in future studies. IAP degradation may also explain the lack of correlation between IAP mRNA and protein levels in rat mesangial cells. Future work must shed light on these regulatory events, to increase our understanding of the different susceptibilities of cells to NO and the role of IAP regulation in glomerulonephritis and mesangial cell function.

Acknowledgments

This study was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 553). We thank Ulrike Müller for expert technical assistance. Drs. Manderscheid and Meßmer contributed equally to this work.

References


