Tumor Necrosis Factor-α Generates Reactive Oxygen Species via a Cytosolic Phospholipase A₂-linked Cascade*

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Reactive oxygen species (ROS) are important regulatory molecules implicated in the signaling cascade triggered by tumor necrosis factor (TNF)-α, although the events through which TNF-α induces ROS generation are not yet well characterized. We therefore investigated selected candidates likely to mediate TNF-α-induced ROS generation. Consistent with the role of Rac in that process, stable expression of RacAsn-17, a dominant negative Rac1 mutant, completely blocked TNF-α-induced ROS generation. To understand better the mediators downstream of Rac, we investigated the involvement of cytosolic phospholipase A₂ (cPLA₂) activation and metabolism of the resultant arachidonic acid (AA) by 5-lipoxigenase (5-LO). TNF-α-induced ROS generation was blocked by inhibition of cPLA₂ or 5-LO, but not cyclooxygenase, suggesting that TNF-α-induced ROS generation is dependent on synthesis of AA and its subsequent metabolism to leukotrienes. Consistent with that hypothesis, TNF-α Rac-dependently stimulated endogenous production of leukotriene B₄ (LTB₄), while exogenous application of LTB₄ increased levels of ROS. In contrast, application of leukotrienes C₄, D₄, and E₄ or prostaglandin E₂ had little effect. Our findings suggest that LTB₄ production by 5-LO is situated downstream of the Rac-cPLA₂ cascade, and we conclude that Rac, cPLA₂, and LTB₄ play pivotal roles in the ROS-generating cascade triggered by TNF-α.

In a variety of non-phagocytic cells, exposure to certain cytokines induces marked transient increases in the intracellular levels of such ROS as O₂⁻ and H₂O₂ (1). For example, exposure to TNF-α or IL-1β increases intracellular levels of ROS in NIH3T3 fibroblasts (2), suggesting that ROS may act as signaling intermediates for TNF-α and IL-1β (3–4). Indeed, these highly reactive molecules are known to regulate many important cellular events in response to TNF-α, including transcriptional factor activation (e.g. NF-κB) (5), cellular proliferation (6), and apoptosis (7–9).

Nonetheless, little is known about the intracellular signaling mediators involved in TNF-induced ROS generation. We previously observed that Rac, a small GTP-binding protein, plays a role in mediating TNF-α-induced activation of c-fos SRE and JNK (10). Similarly, Rac has been suggested to function as regulator of TNF-α-induced intracellular ROS generation (2) and thus appears to mediate TNF-α signaling, at least in part. Still, the precise roles played by Rac and its downstream target molecules in TNF-α signaling to ROS generation remains unknown.

Once activated, Rac in turn activates cPLA₂, which catalyzes the synthesis of AA (11–12). This makes it likely that cPLA₂ is a major downstream mediator of Rac signaling. In addition, we recently observed that metabolism of AA to LTs by 5-LO is involved in Rac signaling to ROS generation and JNK stimulation (13–14). This led us to hypothesize that TNF-α-induced ROS generation is mediated by a Rac-cPLA₂-5LO-linked cascade. To test this hypothesis, we investigated the respective roles played by Rac, cPLA₂, AA, 5-LO, and LTs in the TNF-α-induced generation of ROS.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Plasmids—**DCFDA was obtained from Molecular Probes (Eugene, OR); arachidonic acid, MK-886, indomethacin, AA-861, and AACOCF₃, were from Biomol (Plymouth Meeting, PA); TNF-α, lyso phosphatidic acid, mecaprine, wroughtmann (15) (a PI 3-kinase antagonist), leukotrienes, and NAC were from Sigma. FBS, DMEM, phenol red-free DMEM, gentamicin, and nonessential amino acids were from Life Technologies, Inc. ZK-158252, a specific BLTR antagonist, was kindly from Dr. Claudia Giesen (Experimental Dermatology, Schering AG, Berlin, Germany). All other chemicals were from standard sources and were molecular biology grade or higher. Reporter gene pSRE-luc contains c-fos SRE oligonucleotide sequences (23-mer) inserted into the −53 position of a truncated basal c-fos promoter fused to the luciferase gene (16). pRc-SOD and pCR3.1-Catalase were gifts from Dr. O.S. Kim (National Institutes of Health).

**Cell Culture, Transfections, and Luciferase Assays—**Rat-2 fibroblasts were obtained from the American Type Culture Collection (ATCC, CRL 1764). The cells were grown in DMEM supplemented with 0.1 mM non-essential amino acids, 10% FBS, and penicillin (50 units/ml), streptomycin (50 µg/ml) at 37 °C under a humidified 95:5% (v/v) mixture of air and CO₂. The stable Rat2-RacAsn-17 and Rat2-RacVal-12 clones expressing RacAsn-17, a dominant negative Rac1 mutant, and RacVal-12, a constitutively active form of Rac1, respectively, were described previously (14, 17). Transient transfection was carried out by plating approximately 3 × 10⁵ cells in 60-mm dishes for 24 h and then adding calcium phosphate/DNA precipitates prepared with 6 µg of DNA/dish. To control for variations in cell number and transfection efficiency, all clones

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were co-transfected with 0.3 μg of pCMV-βGal, a eukaryotic expression vector containing the E. coli β-galactosidase (lacZ) structural gene under the transcriptional control of the cytomegalovirus promoter. The quantity of DNA used in each transfection was held constant (6 μg) by adding sonicated calf thymus DNA (Sigma). After incubating 6 h with the calcium phosphate/DNA precipitates, the cells were rinsed twice with PBS before incubating them in fresh DMEM supplemented with 0.5% FBS. Each dish of cells was then rinsed twice with PBS and lysed in 0.1 M of lysis solution (0.2 M Tris (pH 7.6) + 0.1% Triton X-100), after which the lysed cells were scraped and spun for 1 min. Supernatants were assayed for protein concentration and β-galactosidase activities. Luciferase activity was assayed in 10-μl samples of extract using a Luciferase Assay System (Promega) according to the manufacturer’s protocol; luciferase luminescence was counted in luminometer (Turner Design, TD-20/20) and normalized to co-transfected β-galactosidase activity. Transfection experiments were performed in triplicate with two independently isolated sets of cells, and the results were averaged.

Measurement of Intracellular H2O2—Intracellular H2O2 was measured as a function of DCF fluorescence using the procedures of Obha et al. (18). Briefly, cells were grown on coverslips for 2 days and then serum-starved in DMEM supplemented with 0.5% FBS for an additional 2 days. The cells were then stabilized in serum-free DMEM in the presence of 10 ng/ml EGF or LTs for the indicated times. When assessing the effects of inhibitors, cells were pretreated with the respective inhibitor for 30 min. To measure intracellular H2O2, cells were then incubated for 10 min with the H2O2-sensitive fluorophore DCFDA (5 μM), which when taken up fluorescently labels intracellular H2O2 with DCF. The cells were then immediately observed under a laser-scanning confocal microscope using an argon laser, and the evoked emission was filtered with a 515-nm long pass filter. DCF fluorescence was measured in 30 randomly selected cells. Values represent means ± S.D. of DCF fluorescence intensity in three independent experiments. Statistical significance of ROS measurements were assessed with unpaired t test (p < 0.01).

LTB4 Assays—Cells (3 × 105) were plated on 60-mm dishes and incubated in DMEM supplemented with 10% FBS for 24 h. Then the culture media were replaced with DMEM containing 0.5% FBS for additional 24 h, after which they were treated with TNF-α for 30 min. For the measurements of intracellular level of LTB4, the plates were rinsed twice with cold PBS and mixed with four times their volume of absolute ethanol and left at 4 °C for 30 min. The resulting precipitate was removed by centrifugation at 10,000 rpm for 30 min at 4 °C. The supernatant was removed by evaporation under vacuum, and the samples reconstituted in assay buffer were stored under argon at −50 °C until assay for LTB4 using a specific enzyme-linked immunoassorbent assay (Amersham Pharmacia Biotech, RPN 224) as instructed by the manufacturer. The enzyme immunoassay was calibrated with standard LTB4 from 0.31 to 40 pg per well. The sensitivity, defined as the amount of LTB4 needed to reduce zero dose binding, was 0.3 pg/well, which is equivalent to 6 pg/ml. Statistical significance of LTB4 assays were assessed with analysis of variance (p < 0.05).

JNK/STRESS-ACTIVATED PROTEIN KINASE ASSAYS—To assay JNK activity mediated by TNF-α, subconfluent Rat-2 cells were serum-starved for 24 h in DMEM supplemented with 0.5% FBS and then stimulated with TNF-α for 30 min. Each dish of cells was then washed with cold PBS, lysed by incubation for 5 min in 0.5 ml of ice-cold lysis buffer (20 mM Tris (pH 7.4) 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μM leupeptin) with 1 mM PMSF, scraped into Eppendorf tubes and triturated by 10 passes through a 21.1-gauge needle. The supernatant (cell lysate) was then harvested by microcentrifugation at 14,900 rpm for 10 min. Protein concentrations were equalized by normalizing them to the protein levels (assayed by Bradford procedure with Bio-Rad Dye Reagent) measured before the JNK assay.

JNK activity was determined using a JNK assay kit (New England Biolabs) according to the manufacturer’s protocol. Briefly, an amino-terminal c-Jun (amino acid residues 1–89) fusion protein bound to glutathione-Sepharose beads was used to pull down JNK from cell lysates. The kinase reaction (50 μl) was then carried out using the c-Jun fusion protein as a substrate in the presence of cold ATP. Phosphorylation of the c-Jun fusion protein at Ser-63 was measured by Western blot using a polyclonal rabbit anti-phospho-c-Jun antibody that detects only catalytically activated c-Jun phosphorylated at Ser-63.

Protein samples were heated to 95 °C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis on 8% acrylamide gels, followed by transfer to polyvinylidene difluoride membranes for 2 h at 100 V using a NOVEX wet transfer unit. Membranes were then blocked overnight in Tris-buffered saline with 0.01% (v/v) Tween 20 and 5% (w/v) nonfat dried milk, after which they were incubated for 2 h with the primary antibody (anti-phospho-c-Jun) in Tris-buffered saline, and then for 1 h with horseradish peroxidase-conjugated secondary antibody. The blots were developed using enhanced chemiluminescence kits (ECL, Amersham Pharmacia Biotech). Bands on XAR-5 films (Eastman Kodak Co.) corresponding to phospho-c-Jun were measured by densitometry.

RESULTS

Essential Role of ROS in the Activation of c-fos SRE and JNK by TNF-α—To assess the role of ROS in the TNF-α-induced intracellular nuclear signaling cascade, we first examined the effect of NAC, a free radical scavenger, on TNF-α-induced stimulation of c-fos SRE, which is a primary nuclear target of various extracellular agonists (16, 19–20). To accomplish this, Rat-2 cells were transiently transfected with reporter plasmid
pSRE-Luc (3 µg), which contains c-fos SRE oligonucleotides inserted upstream of the c-fos minimal promoter fused to luciferase coding sequences (16). TNF-α-induced SRE activation was monitored by measuring luciferase activities normalized to co-transfected β-galactosidase activity. A 2.9-fold increase in the luciferase activity occurred by TNF-α (10 ng/ml). Pretreatment with NAC dose-dependently inhibited TNF-α-evoked c-fos SRE luciferase activity; for example, 1 mM NAC reduced TNF-α-evoked SRE luciferase activity by ~50% (Fig. 1A). Transient co-transfection of pSOD and pCatalase, expression vectors encoding SOD and catalase, respectively, with pSRE-Luc markedly decreased TNF-α-induced stimulation of SRE-luciferase activity (Fig. 1B). Among the quantities tested, co-transfection with 5 µg of pCatalase reduced TNF-α-induced stimulation of SRE-luciferase activity by ~75%.

The above results strongly suggest the participation of ROS in TNF-α signaling to c-fos SRE. Interestingly, DPI, a flavoprotein inhibitor, had a similar inhibitory effect on TNF-α-induced stimulation of SRE, which suggests that a flavoprotein-binding protein similar to the phagocytic NADPH oxidase may play a role in the signaling pathway from TNF-α to SRE (Fig. 1C). Unlike TNF-α, EGF-induced SRE activation (3.2-fold increase) was little affected.

NAC inhibited TNF-α-evoked JNK activation in a similar fashion. TNF-α (10 ng/ml) induced an ~4.8-fold increase in JNK activity over control, an effect that was dramatically inhibited by pretreatment with NAC (Fig. 2). Moreover, DPI also had a significant inhibitory effect on TNF-α-induced JNK activation, suggesting a flavoprotein-binding protein is also involved in the activation of JNK by TNF-α, and implying a common, essential role for ROS in TNF-α-evoked activation of both JNK and c-fos SRE.

Roles of PI 3-Kinase, Rac, cPLA2, and 5-LO in TNF-α-induced Generation of ROS—The crucial role played by ROS in TNF-α signaling was confirmed by directly measuring TNF-α-induced changes in ROS levels as a function H2O2-sensitive DCF fluorescence. As shown in Fig. 3A, maximum ROS generation (~2.4-fold increase over control) was observed within 15 or 20 min of exposure to TNF-α, after which the levels declined. This effect was previously reported to be Rac-dependent (2), and consistent with that report, we observed that ROS generation was not detectable in Rat2-RacAAO17 cells expressing a dominant negative Rac1 mutant (Fig. 3B) (17).

We recently showed that PI 3-kinase acts upstream of Rac in TNF signaling to JNK or SRE (10). To determine whether this enzyme is involved in ROS generation by TNF-α, we analyzed the effect of wortmannin, a selective inhibitor of PI 3-kinase activity (21–22), and we found that it dramatically inhibited TNF-α-induced ROS generation (Fig. 4). We also recently reported that cPLA2 plays an essential role in mediating Rac signaling to c-fos SRE, JNK, and ROS, thereby acting as an important downstream mediator of Rac (12–14). Thus, one potential mechanism by which Rac may regulate ROS formation in response to TNF-α is via the cPLA2-AA cascade. We therefore assessed the effects of specific inhibitors of cPLA2 or AA metabolism on TNF-α-mediated ROS generation. Pretreatment with 10 µM AACOCF3, a specific cPLA2 inhibitor, almost completely blocked TNF-α-induced ROS generation (>90% inhibition), and similar inhibitory effects were exerted by MK-886, a specific 5-LO inhibitor. This suggests that cPLA2-catalyzed synthesis of AA and subsequent metabolism of AA by 5-LO are involved in the TNF-α signaling to ROS generation. This effect was selective, as levels of ROS were unaffected by 10 µM indomethacin, a nonspecific cyclooxygenase inhibitor (Fig. 4). Apparently, 5-LO, but not cyclooxygenase, plays a crucial role in mediating TNF-α-induced ROS generation by acting downstream of the Rac-AA cascade.

Exogenous LTB4 Induces the Generation of ROS—To investigate further the involvement of 5-LO in TNF-α-induced ROS generation, we assessed the effect of LTB4, products of AA metabolism by 5-LO, on production of intracellular ROS. Exogenous application of LTB4 (0.3 µM) induced maximal increases in

**Fig. 2. Essential role of ROS in JNK activation by TNF-α.** Rat-2 cells were serum-starved and then incubated for 30 min with control buffer or TNF-α (10 ng/ml). Before the addition of agonists, cells were preincubated for 30 min with NAC (1 mM), DPI (5 µM), or control buffer. Protein samples of equal size were then assayed for JNK activity using c-Jun fusion protein (residues 1–89) as a substrate. The results shown are representative of at least three independent transfections.

**Fig. 3. TNF-α stimulates ROS generation in a Rac-dependent manner.** A, Rat-2 cells were serum-starved for 2 days and then stimulated with TNF-α for the indicated times. B, serum-starved Rat-2 and Rat2-RacAAO17 cells were stimulated with the indicated concentrations of TNF-α. DCF fluorescence, reflecting of the relative levels of ROS (arbitrary units), was imaged with a confocal laser scanning fluorescence microscope and then quantified as described under “Experimental Procedures.” Data are expressed as means ± S.D. (n = 30 cells). Statistical significance of ROS measurements was assessed with unpaired t test (p < 0.01).
the levels of ROS; individually, LTC₄, -D₄, or -E₄ had minimal effects, although a LTC₄/D₄/E₄ mixture (0.2 μM) induced an ~3.5-fold increase in the ROS levels; PGE₂ (1 μM) had no effect at all (Fig. 5A). The response to LTB₄ reached a maximum within 3 min and declined thereafter (Fig. 5B). In addition, LTB₄-evoked ROS generation was not inhibited by Rac¹⁷⁻¹⁷ (Fig. 5C), indicating that LTB₄ acts downstream of Rac in the TNF-α signaling pathway leading to the generation of ROS.

Enhanced Generation of LTB₂ by TNF-α in a Rac-dependent Manner—The above results encouraged us to test whether the level of LTB₂ is indeed enhanced by TNF-α in the cells. After incubation in DMEM with 0.5% FBS for 24 h, Rat-2 cells were exposed to TNF-α in control buffer or in the presence of various inhibitors. Consistent with the proposed role of LTB₂ as a downstream mediator, TNF-α significantly elevated levels of LTB₂, an effect that was selectively inhibited by wortmannin, AAOCOF₃, or MK-886 (Fig. 6A). We also measured the extracellular, secreted level of LTB₄ in response to TNF-α and, as shown in Fig. 6B, TNF-α significantly induced the secretion of LTB₄ (e.g. 2.8-fold increase over control buffer at 1 h). The effect of TNF-α on LTB₄ appeared to be critically dependent on Rac activity, as the elevated level of LTB₄ in response to TNF-α is clearly diminished in Rat2-Rac¹⁷⁻¹⁷ cells (17) than in control Rat-2 cells (Fig. 6C). Similarly, the enhanced extracellular LTB₄ level by TNF-α was significantly decreased in Rat2-Rac¹⁷⁻¹⁷ cells than in Rat-2 cells (data not shown). Consistent with the role of Rac1 in LTB₄ production, the basal LTB₄ levels were significantly higher in Rat2-Rac¹⁷⁻¹⁷ cells (14), stably expressing Rac¹⁷⁻¹⁷, a constitutively active form of Rac1, than in control Rat-2 cells (Fig. 6D). Together, our results strongly suggest the mediatory role of Rac1 in the TNF-α signaling pathway to LTB₄ production.

LTB₄ Interaction with BLTR Is Critical for TNF-α Signaling to ROS, JNK, and SRE—It has been suggested that LTB₄ acts in an autocrine manner via BLTR, its cell surface receptor (23–24). We therefore examined the effect of ZK-158252 (Schering AG), a specific BLTR antagonist, on TNF-α-induced ROS generation. As shown in Fig. 7A, pretreatment with 3 μM ZK-158252 completely abolished ROS generation by TNF-α in a manner similar to DPI or NAC (Fig. 7A). As expected, ZK-158252 also inhibited both JNK and SRE stimulation induced by TNF-α (Fig. 7, B and C), suggesting the involvement of BLTR in TNF-α-evoked activation of both JNK and c-fos SRE.

DISCUSSION

The results of the present study indicate that nuclear signaling leading to activation of c-fos SRE or JNK is dependent on generation of ROS, as demonstrated by the inhibitory effects of NAC, catalase, and SOD on TNF-α-induced c-fos SRE or JNK stimulation in Rat-2 fibroblasts. In an attempt to understand better the pathway through which ROS generation is stimulated by TNF-α, we examined the role of the Rac-cPLA₂-linked cascade in that process. First, the role of Rac1 was assessed by comparing ROS generation in Rat-2 and Rat2-Rac¹⁷⁻¹⁷ cells, which revealed that TNF-α acts in an autocrine manner via BLTR, its cell surface receptor (23–24). We therefore examined the effect of ZK-158252 (Schering AG), a specific BLTR antagonist, on TNF-α-induced ROS generation. As shown in Fig. 7A, pretreatment with 3 μM ZK-158252 completely abolished ROS generation by TNF-α in a manner similar to DPI or NAC (Fig. 7A). As expected, ZK-158252 also inhibited both JNK and SRE stimulation induced by TNF-α (Fig. 7, B and C), suggesting the involvement of BLTR in TNF-α-evoked activation of both JNK and c-fos SRE.
direct addition of LTB₄ to the cells (Fig. 5). Consistent with the proposed action of 5-LO being downstream of Rac, LTB₄ generated similar levels of ROS in both Rat-2 and Rat2-Rac₄¹⁷⁻³⁷ cells (Fig. 5C). Although a mixture of cysteinyl LTs (LTC₄/D₄/E₄) also yielded significant increases in ROS (Fig. 5A), addition of any individual cysteinyl LTs did not; thus LTB₄ appears to be the major form of LT responsible for the ROS generation by TNF-α.

Consistent with that role, we were able to detect significant TNF-α- and Rac-dependent increases in the level of LTB₄ in treated cells (Fig. 6). In addition, increasing evidence supports a signaling link between cPLA₂-mediated AA metabolism and ROS generation. For example, Li et al. (28) and Lindsay et al. (29) suggest that LTB₄ activates ROS generation in neutrophils and eosinophils, respectively. Also, an action of LTB₄ through an autocrine stimulatory loop was shown to be crucial for activation of LT synthesis from AA in neutrophils (30). Consistent with that report, we observed a significantly enhanced extracellular secretion of LTB₄ in response to TNF-α (Fig. 6B).

We do not yet know the LTB₄ target molecule(s) mediating ROS generation. Nonetheless, since ZK-158252, a specific BLTR antagonist, markedly inhibited the effects of TNF-α (Fig. 7), we postulate that BLTR, a G protein-coupled, cell surface
The intranuclear transcription factor peroxisome proliferator-activated receptor-α (PPAR-α) plays a role in regulating intracellular signaling cascades leading to the production of LTB₄ and the resultant production of LTB₄ in inflammatory processes (24). In addition, LTB₄ is a potent chemoattractant involved in such inflammatory diseases as bronchial asthma (25), inflammatory bowel diseases (26), and psoriasis (27). Usually, TNF-α-induced production of ROS in non-phagocytes is only 1–2% of that seen in phagocytes, which produce large amounts of O₂⁻ as part of the body's defense against disease processes (31). The generation of ROS in response to TNF-α appears to be involved in TNF-α cytotoxicity (8), and indeed we detected no cytotoxicity under our experimental conditions.2 Instead, our findings with JNK and SRE suggest that ROS generation elicited by TNF-α may play a role in regulating intracellular signaling cascades leading to regulation gene expression (Fig. 7).

In summary, the data presented are indicative of the crucial role played by cPLA₂ and the resultant production of LTB₄ in the regulation of TNF-α-mediated generation of ROS. Although we do not know precisely the downstream mediators of LTB₄, our findings indicate that BLTR is likely involved. Future studies elucidating the linkage between LTB₄ and ROS will likely be pivotal to our complete understanding of TNF-α-evoked intracellular signaling and inflammatory responses.

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