Inhibition of Cytokine Gene Expression by Sodium Salicylate in a Macrophage Cell Line through an NF-κB-Independent Mechanism

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Macrophage-derived cytokines and chemokines are involved at multiple steps of immune and inflammatory responses, and the transcriptional factor NF-κB appears to play a pivotal role in their coordinated upregulation. The anti-inflammatory agents salicylates have been proposed to act in part by inhibiting NF-κB. We have therefore studied the effects of sodium salicylate on lipopolysaccharide (LPS)-induced κB-binding activity and on cytokine and chemokine gene expression in the RAW264.7 murine macrophage cell line and compared them to those of an established NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC). PDTC (100 μM) completely abrogated LPS-induced κB-binding activity and also profoundly inhibited the induction of interleukin 1α (IL-1α), IL-1β, IL-6, IL-10, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and MCP-1 and, to a lesser extent, leukemia inhibitory factor, RANTES, and IL-1Ra. In contrast, sodium salicylate (15 to 20 mM) had no effect on NF-κB activation but, nevertheless, suppressed several LPS-induced cytokine and chemokine genes to a degree similar to that obtained with PDTC. However, compared to PDTC, sodium salicylate caused significantly less inhibition of IL-1Ra and IL-10 gene expression after LPS stimulation. Neither LPS-induced MIP-1α, MIP-1β, nor MIP-2 was significantly affected by PDTC or sodium salicylate, demonstrating that NF-κB is dispensable for the transcriptional regulation of these genes by LPS. In summary, these results suggest that both NF-κB-dependent and NF-κB-independent pathways are necessary for the induction by LPS of a complex cytokine and chemokine response. In the RAW264.7 macrophage cell line, suprapharmacological concentrations of sodium salicylate exert a potent inhibitory effect on LPS-induced cytokine gene induction but appear to accomplish this by interfering with NF-κB-independent pathways of activation.

Monocytes-macrophages are crucial effectors and modulators of the immune response because, in addition to their role in phagocytosis and antigen presentation and processing, they can orchestrate the recruitment, activation, and proliferation of various other effector cell subsets through secretion of potent soluble mediators (39). Among the soluble mediators produced by activated macrophages are cytokines with primarily proinflammatory properties, such as interleukin 1α (IL-1α) and IL-1β (6, 7), and others with generally anti-inflammatory effects, such as IL-1Ra (1) and IL-10 (22). Activated macrophages also produce inflammatory cytokines with potent hematopoietic growth factor activity, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), which have been identified for their roles in proliferation and differentiation of immature hematopoietic cells (26) but which may also directly participate in inflammation, as suggested by the chemotactic, differentiating, as well as growth-enhancing activities that they exert on mature phagocytes (15, 28, 33, 40, 41). Macrophages can also contribute to immune responses through the production of small chemotactic cytokines (chemokines), such as RANTES, MCP-1, MIP-1α, and MIP-2.

A search for common pathways involved in the regulated induction of these diverse gene products has focused on transcriptional control mechanisms and has identified NF-κB as a likely converging point of various immune and inflammatory responses (2). The prototypical and ubiquitous form of functional NF-κB exists as a heterodimer of the p50 and p65 subunits and, under basal conditions, is mostly sequestered in the cytoplasm through its association with an inhibitory IκB subunit (2). Upon activation by various extracellular signals, including bacterial lipopolysaccharide (LPS), multiple and as yet incompletely understood signaling cascades lead to serine phosphorylation of IκB and its proteasome-mediated degradation, resulting in an active p50/p65 complex which migrates to the nucleus (3, 5). Once in the nucleus, NF-κB recognizes specific DNA motifs contained in the 5′ untranslated regions (5′-UTRs) of many inflammatory genes and induces the transcriptional activities of the corresponding promoters.

Inhibitors of NF-κB have been identified and have been found to reduce or abrogate the expression of a variety of inducible inflammatory genes (2). NF-κB inhibitors for the most part comprise antioxidants and are thought to act by preventing the generation of reactive oxygen species, a critical step in several NF-κB-activating pathways (35, 36). Pyrrolidine dithiocarbamate (PDTC), which combines both antioxidant and metal-chelating properties, is a well-studied example of an antioxidant NF-κB inhibitor (21, 24). Among the nonantioxidant molecules reported to inhibit NF-κB activation is sodium salicylate (19), an established anti-inflammatory therapeutic agent. However, the specificity and relevance of NF-κB inhibition as a mechanism of salicylate action have been questioned, particularly in view of the suprapharmacologic and presumably toxic concentrations of salicylates required for these novel effects (9).

To define more precisely the range of inflammatory genes...
which depend on NF-κB for their induction in macrophages and to examine the potential relevance of NF-κB inhibition to the anti-inflammatory actions of salicylates, we compared the effects of sodium salicylate and the established NF-κB inhibitor PDTC on LPS-induced NF-κB activation and cytokine induction using gel retardation and RNase protection assays, respectively.

MATERIALS AND METHODS

Reagents. PDTC and sodium salicylate were obtained from Sigma (St. Louis, Mo.). Stock solutions of appropriate concentrations were prepared in sterile, pyrogen-free water and were filter sterilized.

Cells. The RAW264.7 mouse monocyte-macrophage cell line was obtained from the American Type Culture Collection repository (Rockville, Md.) and was maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, Md.) supplemented with penicillin, streptomycin, and 10% fetal bovine serum (Hyloclone, Logan, Utah) on 60-mm tissue culture dishes. For stimulation, cells were preincubated with sodium salicylate (15 or 20 mM), PDTC (100 μM), or phosphate-buffered saline for 1 h prior to the addition of LPS. Identically treated duplicate dishes were used for preparation of nuclear extracts and RNA.

Preparation of nuclear extracts. Nuclear extracts were prepared essentially as described previously (4), with modifications. Briefly, cells grown in 60-mm dishes were washed once in phosphate-buffered saline and were then allowed to swell descriptively (4), with modifications. Briefly, cells grown in 60-mm dishes were washed once in phosphate-buffered saline and were then allowed to swell.

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PDTC and sodium salicylate and was examined by multiprobe RNase protection assays. This technique allowed us to perform a quantitative analysis of multiple genes simultaneously, including those for inflammatory cytokines, chemokines, and hematopoietic growth factors. To obtain optimal images of the gels, PhosphorImager settings that allowed the visualization of all relevant transcripts, including those expressed very weakly, were chosen. Since these optimal settings were accompanied by some loss of linearity of the visual signal intensity, we also performed quantitative analysis of the gels.

In our initial RNA studies, we examined the expression of the genes for the pleiotropic inflammatory cytokines IL-1α and IL-1β, their natural antagonist IL-1Ra, and the anti-inflammatory cytokine IL-10. MIF was also examined in this assay. As shown in Fig. 2a, LPS induced marked upregulation of IL-1β gene expression and, to a lesser degree, IL-1Ra, IL-1α, and IL-10 gene expression. In contrast, MIF was constitutively expressed at a high level in this cell line and was not substantially affected by LPS stimulation. Pretreatment with PDTC caused a marked suppression of LPS-induced cytokine gene expression. The signal intensity for each gene of interest was quantitated on the PhosphorImager with ImageQuant software, and it was normalized to that of the L32 housekeeping control to adjust for small RNA loading variations. As shown in Fig. 2b, inhibition by PDTC ranged from 72% for IL-1Ra to 100% for IL-10. Compared to PDTC, sodium salicylate exhibited a similarly potent suppression of inducible IL-1α and IL-1β gene expression, but it had a much more modest effect on IL-10 and IL-1Ra gene induction (73 and 7%, respectively). MIF gene expression was high at the baseline and was unaffected by either PDTC or sodium salicylate.

In order to examine whether the suppression of LPS-induced cytokine gene expression observed with sodium salicylate might be relevant to the known anti-inflammatory properties of salicylates, we repeated these experiments using lower concentrations of salicylate. As shown in Fig. 2c, the suppressive effect of sodium salicylate on IL-1α and IL-1β gene induction was significantly more prominent at a concentration of 20 mM than at a concentration of 15 mM. At pharmacological concentrations of 3 mM, sodium salicylate had no effect on LPS-induced cytokine gene expression (data not shown). Because the higher concentrations of salicylates resulted in markedly increased toxicity after prolonged incubations, we continued our studies using a concentration of 15 mM.

Parallel studies performed with a transiently transfected reporter gene construct driven by the murine IL-1β promoter suggested that the effects of both PDTC and salicylate on IL-1β gene expression resulted from inhibition of promoter activity (19a).

Suppression of LPS-induced chemokine and hematopoietic growth factor gene expression by PDTC and sodium salicylate. In order to more broadly define the subset of inflammatory genes susceptible to inhibition by PDTC and salicylates, we studied the effects of these two compounds on LPS-induced chemokine and hematopoietic growth factor gene expression in the RAW264.7 cell line. As shown in Fig. 3 and 4, treatment with LPS induced the intense expression of a broad range of hematopoietic growth factor and chemokine genes including those for GM-CSF, G-CSF, LIF, RANTES, MIP-1α, MIP-1β, MIP-2, and MCP-1, as well as modest levels of IL-6 mRNA. Pretreatment with PDTC caused a striking inhibition of GM-CSF, G-CSF, LIF, RANTES, and MCP-1, whereas it did not significantly affect the induction of MIP-1α, MIP-1β, or MIP-2 mRNA. Sodium salicylate exerted a similar suppressive effect on hematopoietic growth factor and chemokine gene expression, but compared to PDTC, it caused slightly less suppression of G-CSF. Moreover, a prominent band of approximately 150 bp was consistently suppressed by PDTC but not by salicylate (Fig. 4a). This band migrated more slowly than the TCA-3 protected probe (Fig. 4a, lane 1) and was not observed in an assay performed with only the MCP-1 probe (Fig. 4b). Thus, it is likely that this additional band represented an alternatively
FIG. 3. Effects of PDTC and sodium salicylate on LPS-induced hematopoietic growth factor gene expression. RNA obtained from RAW264.7 cells as described in the legend to Fig. 2 was subjected to an RNase protection assay with a panel of radiolabeled hematopoietic growth factor RNA probes as described in Materials and Methods. (a) Autoradiograph of the RNase protection assay. Lane 1, unstimulated cells; lane 2, cells treated with LPS alone; lane 3, cells treated with LPS plus PDTC at 100 μM; lane 4, cells treated with LPS plus salicylate at 15 mM; lane 5, control RNA from cells transfected with the murine IL-6 cDNA (obtained from Pharmingen); lane 6, yeast tRNA as a negative control for nonspecific hybridization (ns); lane 7, unhybridized and undigested RNA probes. Note that the sizes of the undigested probes are slightly greater than the positions of the corresponding protected probes because of noncomplementary overhangs. (b) Quantification of the results shown in panel a obtained with the ImageQuant software. Transcript intensity is represented relative to that of the L32 housekeeping control.

spliced variant of one of the chemokine genes other than those for TCA-3 and MCP-1. However, its exact identity remains to be determined.

DISCUSSION

We have examined the effects of PDTC and sodium salicylate on LPS-induced kB-binding activity as well as cytokine and chemokine gene expression in the well-characterized RAW264.7 murine macrophage cell line. In this system, intense activation of NF-kB correlated with high-level expression of multiple cytokine and chemokine transcripts. We found that whereas 100 μM PDTC completely inhibited LPS-induced kB-binding activity, concentrations of sodium salicylate up to 20 mM failed to inhibit this activity. PDTC also caused an almost complete suppression of IL-1β, IL-1α, IL-6, IL-10, GM-CSF, G-CSF, and MCP-1 gene induction and, to a lesser extent, LIF, RANTES, and IL-1Ra gene induction after LPS stimulation. These observations supported a role for NF-kB in the upregulation of a broad range of potentially pathogenic immune and inflammatory mediators. On the other hand, PDTC had virtually no effect on MIP-1α, MIP-1β, or MIP-2 induction.

Remarkably, sodium salicylate was devoid of any inhibitory effect on kB-binding activity but was nevertheless a potent suppressor of LPS-induced cytokine and chemokine gene expression. The cytokine inhibitory effect of sodium salicylate was observed only at suprapharmacological concentrations (15 to 20 mM). Most cytokine genes inhibited by PDTC were suppressed to a similar degree by sodium salicylate. However, the induction of two anti-inflammatory genes, those for IL-1Ra and IL-10, was relatively unaffected by salicylate. Indeed, IL-1Ra and IL-10 were suppressed to a much lower degree by sodium salicylate than by PDTC. Thus, the contrasting effects of PDTC and sodium salicylate on NF-kB correlated with their differential effects on two anti-inflammatory cytokines.

The finding that the NF-kB inhibitor PDTC suppressed the induction of several cytokine genes confirmed previous reports regarding the importance of kB-like sequences in mediating the transcriptional activities of specific cytokine and chemokine promoters including those of IL-1β (14), IL-1Ra (16, 32), G-CSF (13), GM-CSF (13), RANTES (25), and MCP-1 (10). Similarly, the lack of suppression of MIP-1α and MIP-1β by PDTC observed in the current study was consistent with the absence of consensus kB-binding sites in the 5’ untranslated region of either gene (42) and suggested that the transcriptional regulation of both MIP-1 genes is entirely independent of NF-kB. In contrast, the lack of suppression of MIP-2 by PDTC was surprising since MIP-2 is a homolog of the three human GRO(α-γ) proteins and is a close relative of IL-8, and all four of these appear to be NF-kB-regulated chemokines (23, 24). Indeed, the murine MIP-2 promoter itself had been studied in RAW264.7 cells with sequentially truncated reporter gene constructs. These studies suggested a critical role for a kB consensus binding site in conferring LPS responsiveness to the MIP-2 promoter in reporter gene assays (42). However, in light of our results, it is likely that other, presumably non-kB enhancer sequences are sufficient for induction of the endogenous MIP-2 gene by LPS.

In the current study, we observed a suppressive effect of sodium salicylate on the induction of inflammatory genes in vitro, but only at suprapharmacological concentrations of 15 mM or more, suggesting that these inhibitory effects may not be directly relevant to the known anti-inflammatory effects of salicylates in vivo. The observation that high concentrations of salicylate can suppress the expression of specific cytokine or chemokine genes had been previously reported by others. For instance, Gautam et al. (11) demonstrated the suppression of the chemokines IP-10 and MCP-1 by 20 mM salicylate in bone marrow stromal cells stimulated with IL-1α. In that study, as well as in studies performed with phorbol ester-treated Jurkat T cells (19), LPS-stimulated pre-B cells (19), and glutamate-treated primary neurons (12), suppression of gene expression
or cell toxicity was associated with suppression of NF-κB activation.

Thus, the lack of NF-κB inhibition by sodium salicylate observed in the current study was surprising given the comparable time courses and salicylate concentrations that we used. However, our findings are consistent with those of Farivar and Brecher (8) and Xia et al. (43), who reported the suppression by sodium salicylate of inducible nitric oxide synthase (iNOS) mRNA in primary cardiac fibroblasts and P-selectin mRNA in primary endothelial cells, respectively, independently of any effect on NF-κB. Moreover, Takashiba et al. (37) found that 20 mM sodium salicylate suppressed an LPS-induced NF-κB-independent DNA-binding activity of unknown identity. This putative transcriptional regulator was suggested to be important for inducible transcriptional activity of the human tumor necrosis factor alpha gene. The apparent discrepancies between the results of various studies of salicylate actions may thus reflect the specificities of the cell types and stimuli used. Nevertheless, our results strongly suggest that mechanisms other than NF-κB inhibition can be invoked to explain at least some of the reported effects of salicylates on cytokine gene expression.

The mechanisms for NF-κB-independent suppression of cytokine gene expression by salicylates remain to be identified. Presumably, NF-κB-independent transcriptional regulators are modulated by salicylates either directly or indirectly. It is becoming clear that salicylates affect several discrete signaling pathways. For instance, it has been reported that salicylates induce the DNA-binding activity of the heat shock transcription factor (17). More recently, Schwenger et al. (29–31) found that sodium salicylate inhibited tumor necrosis factor-induced but not epithelial growth factor-induced p42-p44 mitogen-activated protein kinases (MAPKs) and stress-activated protein kinase, while it activated p38 MAPKs. Notably, p38 MAPK activation was associated with decreased phosphorylation and degradation of IkB and with the induction of apoptosis. The direct targets of salicylate involved in these reported effects have not been precisely identified, however. Indeed, it has been suggested that salicylates may not act through specific targets but, rather, exert a generalized inhibitory effect on cellular kinases (9), which could explain their inhibitory effects on a broad range of cellular events.

The current study did not directly address whether the observed suppression of cytokine gene induction by either salicylate or PDTC was the result of decreased transcriptional rates or of reduced mRNA stability. However, in other experiments, we were able to demonstrate that both salicylate and PDTC suppress LPS-induced murine IL-1β promoter activity in reporter gene assays (19a), consistent with other results demonstrating that the suppressive effects of PDTC and salicylate on gene expression were the result of interference at the level of transcription rather than at the level of mRNA stability (24).

In summary, the present study defines a set of macrophage-derived inflammatory mediators requiring NF-κB for their induction by bacterial LPS. These mediators include the cytokines and hematopoietic growth factors IL-1α, IL-1β, IL-1Ra, IL-6, IL-10, GM-CSF, G-CSF, and LIF and the chemokines RANTES and MCP-1. Sodium salicylate is also shown to inhibit most of these inflammatory mediators markedly, but through an NF-κB-independent mechanism. Interestingly, sodium salicylate does not appear to inhibit the expression of the anti-inflammatory cytokine IL-1Ra. Further characterization of the signaling pathway(s) susceptible to inhibition by suprapharmacological concentrations of salicylates could lead to important insights into the regulation of inflammatory and immune responses.

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