Synergistic Induction of NR4A Orphan Nuclear Receptors by LPS and cAMP in Murine Macrophage Cells

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Key words: Nur77; Nurr1; NOR1; gene regulation

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ABSTRACT

Although NR4A orphan nuclear receptors have been implicated in inflammatory gene expression and atherosclerosis, there is little information regarding their regulation by combinations of stimuli likely to be found in vivo, such as LPS and inducers of cAMP. LPS rapidly induced Nur77 and NOR1 mRNAs but had little effect on expression of Nurr1 mRNA. All three NR4As were rapidly induced by 8-bromo-cAMP and remained elevated for at least 8 h. Maximum induction of NOR1 by 8-bromo-cAMP was much greater than with LPS, but maximum induction of Nur77 was similar with both agents. Whereas Nurr1 mRNA had very little response to LPS, it was strongly induced by 8-bromo-cAMP, and Nurr1 mRNA levels remained elevated for at least 20 h. The combination of 8-bromo-cAMP and LPS acted synergistically to strongly induce NOR1 and Nur77, whereas LPS partially inhibited the induction of Nurr1 by 8-bromo-cAMP. Nurr1 protein levels correlated with Nurr1 mRNA levels but exhibited slower response kinetics. In summary, the NR4A receptors did not respond identically to individual stimuli or to combinations of stimuli. In particular, the magnitude of the responses to combinations of stimuli was quite different than to individual stimuli. Thus, NR4A receptor expression and the resulting functional consequences are more complex than appreciated from previous studies that evaluated regulation of NR4A expression by single stimuli.

INTRODUCTION

The NR4A family of orphan nuclear receptors is comprised of three members: Nur77 (NR4A1; also known as TR3 and NGFI-B), Nurr1 (NR4A2; also known as NOT), and NOR1 (NR4A3; also known as MINOR). Initially identified more than ten years ago as early response genes to growth factors [1-3], these proteins are expressed in many different tissues and cell types, including liver, kidney, heart, skeletal muscle, brain, and macrophages [4-6]. Members of the NR4A family can activate gene expression in a ligand-independent manner, but the identities of target genes in many cells remain largely unknown. NR4A proteins can bind to their cognate DNA binding sites as monomers, homodimers, as heterodimers with other NR4A family members or—in the case of Nur77 and Nurr1—as heterodimers with RXR [4, 6].

Because NR4A receptors have been implicated as regulators of inflammatory gene expression in macrophages and as factors in atherosclerosis [7-12], there has been increased interest in their roles and regulation in macrophages. Previous studies demonstrated induction of NR4A receptors in macrophages by LPS, TNFα, interferon-γ, IL-1β, and oxidized low density lipoprotein [10]. During inflammation or infection in vivo, however, macrophages are exposed to multiple stimuli, which can include agents such as catecholamines or prostaglandins that stimulate intracellular cAMP synthesis, and the responses of specific genes to combinations of stimuli can vary significantly from their responses to individual stimuli. NR4A expression is induced by cAMP in liver, muscle and pituitary cells [13-16], but it has not been determined previously whether cAMP also induces expression of any of the NR4A isoforms in macrophages or, perhaps more importantly, whether cAMP alters their responses to inflammatory stimuli such as LPS. Therefore, this study was conducted to evaluate the potential impact of cAMP, alone or in combination with LPS, on expression of NR4A family members in macrophage cells.

MATERIALS AND METHODS

Cell culture. The RAW 264.7 murine macrophage cell line (American Type Culture Collection, Rockville, MD) was maintained in DMEM high glucose media (Cambrex Bioscience, Walkersville, MD) containing 15 mM HEPES (pH 7.5), 4 mM glutamine, 10% fetal calf serum (HyClone Laboratories, Logan, UT), penicillin (200 U/ml) and streptomycin (200 μg/ml) at 37 ^{o}C in a 5% CO_2/air mixture. Cells were stimulated with 100 ng/ml LPS (TLR grade; Axxora, LLC, San Diego, CA), 0.5 mM 8-bromo-cAMP (Sigma, St. Louis, MO) or a combination of the two agents.

Isolation and analysis of RNA. Total cellular RNA was isolated and analyzed by real time PCR as described [17]. NR4A nuclear receptor mRNA level were measured by using SYBR Green PCR Master Mix (Applied Biosystems, Foster city, CA) on an ABI 7700 sequence-detection system (Applied Biosystems). Sequences of the primer pairs used for quantification of NR4A mRNAs by real time PCR in this study were as described in Supplementary Table 1 of Pei et al. [16]. NR4A mRNA levels in each sample were normalized to the levels of 18S rRNA as determined by quantitative real-time PCR. Relative changes in gene expression were calculated by the ΔΔCt method [18].

Western blot analysis. Cells were washed with PBS and lysed in RIPA buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride, 1mM dithiothreitol , 1mM Na_3VO_4, 50 mM NaF and complete mini protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). BCA protein assay kit from Pierce (Rockford, IL) was used to quantify the proteins. Forty-μg aliquots of whole cell lysates were resolved by 10% SDS-PAGE and transferred to a Hybond nitrocellulose membrane (Amersham Biosciences Corp, Piscataway, NJ). Equal loading of samples was confirmed by staining the membrane with SYPRO Ruby protein blot stain (Molecular Probes, Eugene, OR) prior to immunoblotting. Immunodetection of Nurr1 was done with a rabbit polyclonal antibody (sc-991) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Proteins were visualized using anti-rabbit horseradish peroxidase conjugated antibody (Amersham Biosciences) and the Western Lightening Chemiluminescence Reagent Plus detection system (Perkin Elmer Life Sciences, Boston, MA).

RESULTS AND DISCUSSION

Although Northern blots showing induction of Nur77 and NOR1 mRNAs by LPS in RAW 264.7 cells have been presented [10], quantification of NR4A mRNA levels in response to LPS has not been reported for these cells. In agreement with previous Northern blot results, mRNAs for Nur77 and NOR1 increased rapidly and peaked at 1 and 2 h, respectively, following LPS treatment (Figure 1). The Nur77 induction profile is very similar to that seen for LPS-treated primary murine macrophages (Supplemental Figure 3 of Barish et al.[5]). NOR1 mRNA levels peaked at 2 h and rapidly declined by 4 h (Figure 1), whereas NOR1 mRNA was highly elevated at both 2 and 4 h in primary murine macrophages [5]; the induction profiles were otherwise similar in both primary macrophages and in RAW 264.7 cells. Unlike the responses of Nur77 and NOR1 mRNAs, however, LPS had little effect on Nurr1 mRNA. This is in dramatic contrast to the robust induction of Nurr1 mRNA by LPS in primary macrophages, which exhibited a delayed response, peaking at 16 h [5]. The delayed response suggests that Nurr1 induction may be a secondary response to an autocrine factor(s) that is induced by LPS in primary macrophages but is absent in RAW 264.7 cells.

Unlike the case with LPS, mRNAs for all three NR4As were induced by 8-bromo-cAMP (Figure 2). As in their response to LPS, levels of Nur77 and NOR1 mRNAs peaked at 1 and 2 h, respectively. In contrast to the responses to LPS, however, the mRNA levels did not very rapidly return to baseline but declined more slowly and remained somewhat elevated for at least 8 h. These response profiles are similar to the NR4A responses to 8-bromo-cAMP in murine hepatocytes [16] but differ from the rapid return to baseline for NOR-1 mRNA following treatment with a β-adrenergic agonist in a mouse myoblast line [13]. Maximum fold-induction of NOR1 by 8-bromo-cAMP was much greater than with LPS, but maximum fold-induction of Nur77 was similar with both agents (Figure 2). Whereas Nurr1 mRNA had very little response to LPS, it was strongly induced by 8-bromo-cAMP, peaking around 2 h, and the mRNA levels remained elevated for at least 20 h (Figure 2). As an example of the potential relevance of these results to inflammatory disease, Nurr1 in human synoviocytes was found previously to be strongly induced by the cAMP-elevating agents prostaglandin E2 or forskolin, acting via a CRE sequence in the Nurr1 promoter [19].

Simultaneous treatment with 8-bromo-cAMP clearly modified the responses of all three NR4As to LPS, although not in an identical fashion. In the case of Nur77 and NOR1, the

response to both agents was synergistic, essentially resulting in an amplification of the peak portion of the induction profile (Figure 3). By 4 h, the relative mRNA levels for Nur77 and NOR1 were indistinguishable for cells treated with either 8-bromo-cAMP or 8-bromo-cAMP plus LPS. The response of Nurr1 to the combination of both agents was quite different to the responses of Nur77 and NOR1. Whereas the induction of Nurr1 mRNA by 8-bromo-cAMP or 8-bromo-cAMP plus LPS was virtually identical within the first hour, a significant inhibitory effect of LPS was readily apparent by 2 h and sustained through at least 8 h (Figure 3). Based on the minimal response of Nurr1 to LPS alone, the inhibitory effect of LPS on the induction of Nurr1 by 8-bromo-cAMP at longer times was not anticipated. This unexpected effect further illustrates the importance of characterizing NR4A responses to combinations of inflammatory stimuli.

Western blotting was used to compare induction of Nurr1 protein (Figure 4) with induction of Nurr1 mRNA (Figure 3). Nurr1 protein was undetectable in untreated cells and in cells treated with LPS alone. Consistent with the strong induction of Nurr1 mRNA, Nurr1 protein became detectable at 8 h in cells treated with 8-bromo-cAMP, either alone or in combination with LPS, and was most strongly expressed at 16 and 24 h (Figure 4). Although exhibiting slower response kinetics, the pattern of changes in levels of Nurr1 protein correlated well with the pattern of changes in Nurr1 mRNA levels. At 24 h, levels of Nurr1 protein in cells treated with LPS plus 8-bromo-cAMP were consistently somewhat lower than in cells treated with 8-bromo-cAMP alone. Attempts to also monitor protein levels of Nur77 and NOR-1 with commercially available antibodies were unsuccessful. Whether this was due to low levels of these proteins in RAW 264.7 cells or to inadequate sensitivity of the antibodies is unclear.

NR4A receptors are expressed in wide variety of cell types and tissues, including brain, liver, muscle, pituitary cells, adipose tissue, kidney, and macrophages [4, 6]. Because they function in a ligand-independent manner, changes in NR4A expression are a major determinant of their activity as transcriptional regulators. Accordingly, the identification of stimuli that regulate NR4A expression and the elucidation of patterns of changes in NR4A expression are essential for developing an understanding of the functions of the NR4A family members. Studies using cultured cells frequently evaluate responses only to individual stimuli. However, cells are simultaneously exposed in vivo to multiple stimuli and can respond in ways that may not be predictable from responses to single stimuli in cultured cells. Because NR4A receptors

have been implicated as regulators of inflammatory gene expression in macrophages and as factors in inflammatory diseases such as atherosclerosis and arthritis [7-12, 20, 21], there has been increased interest in regulation of their expression by various inflammatory stimuli, including LPS [10]. Several studies have shown that NR4A expression can be induced by cAMP [13-16, 19], whose intracellular levels can be increased in response to mediators of inflammation or stress such as prostaglandins or catecholamines. However, potential interactions between LPS and cAMP analogs or cAMP-elevating agents in regulating NR4A expression have not been reported previously. The present results indicate that the regulated expression of NR4A members within macrophages in vivo and the resulting functional consequences are likely to be significantly more complex than appreciated from previous studies that evaluated regulation of NR4A expression by single stimuli in cultured cells. In addition to inducing NR4A expression, the cAMP/protein kinase A pathway can enhance transcriptional activity of NR4A receptors independently of changes in NR4A levels [14, 22], thus potentially adding yet another layer of regulatory complexity.

Very few genes that are directly regulated by NR4A receptors have been identified for any cell type. Potential target genes of NR4A receptors in macrophages have recently been identified by microarray analysis of stable transfectants of RAW 264.7 cells overexpressing individual NR4A receptors, and several of these are downstream effectors of inflammatory signaling molecules [11]. However, sorting out specific consequences of changes in NR4A expression in macrophages following stimulation will pose a considerable challenge for future studies not only because of the simultaneous expression of all three NR4A family members with potentially redundant activities but also because potential target genes may be independently regulated by cAMP and/or LPS.

ACKNOWLEDGEMENTS

This research was supported in part by NIH grants RO1 GM57384 and RO1 GM064509 to SMM.

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FIGURES

FIGURE 1. Responses of NR4A mRNAs to LPS. RAW 264.7 cells were exposed to 100 ng/ml LPS for the times indicated. Levels of mRNAs are expressed relative to the mean value for untreated control cells. Results are averages ± SEM for three independent experiments.

FIGURE 2. Induction of NR4A mRNAs by 8-bromo-cAMP. RAW 264.7 cells were exposed to 0.5 mM 8-bromo-cAMP for the times indicated. Levels of mRNAs are expressed relative to the mean value for untreated control cells. Results are averages ± SEM for three independent experiments.

FIGURE 3. Responses of NR4A mRNAs to 8-bromo-cAMP plus LPS. RAW 264.7 cells were exposed to a combination of 8-bromo-cAMP and LPS for the times indicated. Levels of mRNAs are expressed relative to the mean value for untreated control cells. To facilitate comparisons, responses to LPS or 8-bromo-cAMP alone are included on these graphs. Results are averages ± SEM for three independent experiments.

FIGURE 4. Induction of Nurr1 protein by LPS, 8-bromo-cAMP, or a combination of both agents. Western blotting to detect Nurr1 protein was performed on lysates of cells treated for the indicated numbers of hours with the various stimuli. Nurr1 is indicated by the arrow, and untreated control cells are designated as C. The nonspecific band of higher molecular weight served as an internal loading control. Results are representative of three independent experiments.

FIGURE 1

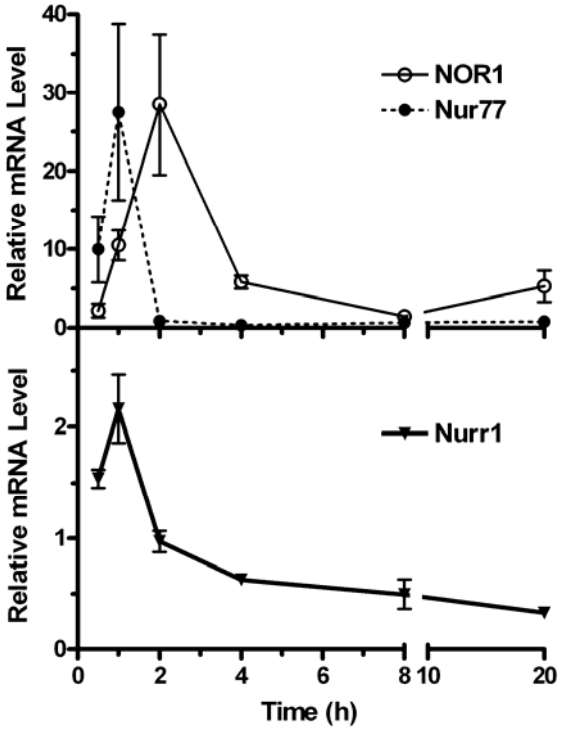

FIGURE 2

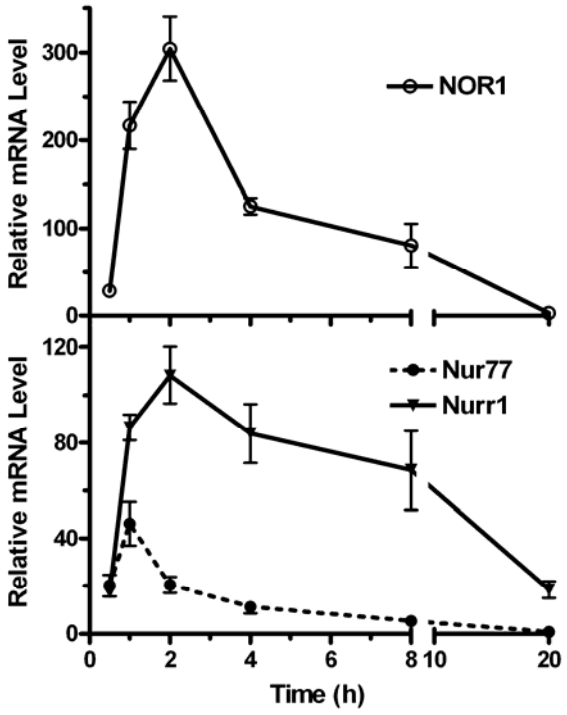

FIGURE 3

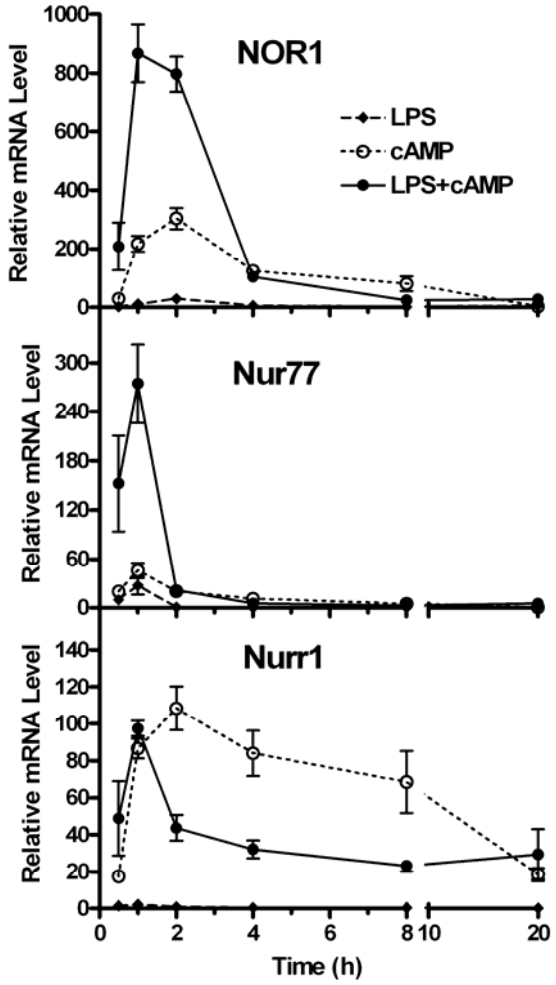

FIGURE 4