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Role of Heat Shock Protein 90 Dissociation in Mediating Agonist-Induced Activation of the Aryl Hydrocarbon Receptor

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ABSTRACT

The aryl hydrocarbon receptor (AhR) is a cytosolic basic helix-loop-helix protein that associates with a chaperone complex that includes two molecules of heat shock protein 90 (HSP90). It has been hypothesized that after ligand binding, the AhR dissociates from its chaperone complex and translocates into the nucleus, where it heterodimerizes with its DNA binding partner, the AhR nuclear translocator (ARNT), and activates specific genes. However, it remains unclear whether nuclear translocation of the AhR occurs before or after dissociation of the HSP90/chaperone complex. Because sodium molybdate stabilizes the AhR-HSP90 interaction and inhibits the gene activation of a number of steroid receptors, we reasoned that molybdate would be a useful tool in delineating the role of HSP90 dissociation in AhR nuclear translocation. In this study, we demonstrate that molybdate inhibits AhR gene activation in both HepG2 and Hepa-1 cells in a concentration-dependent manner and protects the AhR against agonist-induced proteolysis. In addition, we demonstrate that AhR/ARNT dimerization, but not nuclear translocation of the AhR, is inhibited by molybdate. This indicates that 1) HSP90 dissociation is not required for nuclear translocation of the AhR, 2) HSP90 dissociation is essential for formation of the AhR/ARNT heterodimer, and 3) an additional undefined regulatory step is required for AhR/ARNT dimerization in the nucleus.

Polyhalogenated aromatic hydrocarbons are ubiquitous environmental contaminants that have been characterized as potent toxicants and carcinogens. Many of these compounds, such as 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD), elicit diverse biological effects on animal models, including a suppressed immune response, impaired reproduction, and promotion of carcinogenesis. The biological risk of TCDD has been correlated with its ability to bind and activate the aryl hydrocarbon receptor (AhR) (for a review, see Hankinson, 1995).

The AhR is a member of the basic helix-loop-helix/PAS protein family. Additional members of this family include its dimerization partner, the AhR nuclear translocator [ARNT; also referred to as hypoxia inducible factor (HIF) 1β], HIF-1α [which regulates the hypoxic response (Wang et al., 1995)], a number of proteins that are involved in regulating circadian rhythms, Sim [a protein involved in *Drosophila melanogaster* neurogenesis (Muralidhar et al.,1993)], and coactivator proteins such as Src-1 (Onate et al.,1995). Of these proteins, only AhR, HIF-1α, and Sim interact with heat shock protein 90 (HSP90) (McGuire et al., 1995; Gradin et al., 1996). The characteristic feature of this protein family, the PAS domain, facilitates ligand binding and dimerization of the AhR as well as its association with a number of additional proteins, including HSP90 (Antonsson et al., 1995; Fukunaga et al., 1995; Perdew and Bradfield, 1996).

In the absence of ligand, the AhR exists as a 9S cytosolic complex that is composed of two molecules of HSP90 and a novel immunophilin known as AhR associated protein 9 or AhR inhibitory protein (Chen and Perdew, 1994; Carver and Bradfield, 1997; Ma and Whitlock, 1997). HSP90 has been shown to be an essential component of the AhR signaling pathway (Carver et al., 1994; Whitelaw et al., 1995) and its association with the unliganded AhR has been thought to increase the ability of the AhR to bind ligand and inhibit its nuclear translocation and DNA binding (Pongratz et al., 1992; Ikuta et al., 1998). The DNA binding form of the AhR, the 6S AhR/ARNT heterodimer, activates a number of genes involved in xenobiotic metabolism, such as *CYP1A1* and *CYP1A2*. In addition to activating gene transcription, agonist

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ABBREVIATIONS: TCDD, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; PAS, Per/ARNT/Sim (periodicity/aryl hydrocarbon receptor nuclear translocator/simple-minded); HIF, hypoxia inducible factor; ARNT, aryl hydrocarbon receptor nuclear translocator; HSP90, 90-kDa heat shock protein; GR, glucocorticoid receptor; IP, immunoprecipitation; EMSA, electrophoretic mobility shift assay; DRE, dioxin responsive element; TTBS, Tris-buffered saline/Tween 20; BNF, β-naphthoflavone; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide.

binding of the AhR results in a rapid depletion of the AhR protein (Giannone et al., 1998; Roman et al., 1998).

Although HSP90 has been shown to be an essential component of the AhR signaling pathway, its role in AhR nuclear translocation remains unclear. For example, both the detection of the AhR in the nuclei of a number of distinct cell types in the absence of ligand (RP, unpublished observations) and the detection of an HSP90- associated form of the AhR in the nuclei after exposure to agonist (Wilhelmsson et al., 1990; Perdew, 1991) imply that HSP90 dissociation may not be required for AhR nuclear import. In contrast, deletion of the PAS domain of the AhR has been shown to result in ligand-independent nuclear translocation of the AhR, which implies that it is the association of HSP90 with the PAS domain that prohibits the unliganded AhR from accessing the nucleus (Ikuta et al., 1998). Thus, based on these data, at least two possibilities are likely: dissociation of HSP90 may regulate both nuclear import of the AhR and its subsequent dimerization with ARNT, or dissociation of HSP90 may regulate dimerization with ARNT in the nucleus, whereas AhR nuclear translocation may be regulated by some undetermined yet dynamic mechanism.

The role of HSP90 in mediating nuclear translocation of its associated proteins has been extensively studied using the glucocorticoid receptor (GR) signaling pathway as a paradigm (for a review, see Pratt and Toft, 1997). However, the current data available seems to be conflicting or at least complex. First, the addition of a nuclear localization signal to HSP90 that was associated with a GR lacking its native nuclear localization signal resulted in nuclear localization of the GR-HSP90 complex in the absence of agonist. This indicates that only the unmasking of a nuclear localization signal, but not HSP90 dissociation, may be required for nuclear import (Kang et al., 1994). The use of sodium molybdate to prevent GR-HSP90 dissociation in vivo, however, demonstrated that the GR-HSP90 associated complex is resistant to nuclear translocation in the presence of agonist, presumably by preventing the dynamic association/dissociation kinetics between the respective proteins (Yang and DeFranco, 1996). Finally, a more recent study has demonstrated that both the unliganded, HSP90-associated and liganded HSP90-free forms of the GR traffic across the nuclear membrane, indicating a complex yet dynamic role for HSP90 in GR nuclear translocation (Hache et al., 1999).

Because the HSP90-associated form of the AhR is stabilized by molybdate in a manner similar to that of the GR (Denison et al., 1986; Cuthill et al., 1987; Manchester et al., 1987; Roberts et al., 1990; Dolwick et al., 1993a), we have used molybdate as a tool to test the hypothesis that dissociation of HSP90 from the AhR is a prerequisite to AhR nuclear translocation. In this study, we have shown that molybdate inhibits AhR gene activation in a concentration-dependent manner and protects the AhR against agonist-induced proteolysis. In addition, we demonstrate that AhR/ARNT dimerization, but not nuclear translocation of the AhR, is inhibited by molybdate, which indicates that 1) HSP90 dissociation is not required for nuclear translocation of the AhR, 2) HSP90 dissociation is essential for formation of the AhR/ARNT heterodimer, and 3) an additional, undefined regulatory step is required for AhR/ARNT dimerization in the nucleus.

Experimental Procedures

Oligonucleotides. Oligonucleotides were purchased from Life Technologies (Gaithersburg, MD). The annealed oligonucleotides that were used as the radiolabeled probe for the electrophoretic mobility shift assays (EMSAs) that contained the dioxin response element (DRE; underlined) are 5′-TCGAGCTGGGGGCATTGCGTGACATTAC (OL-17) and 3′-TCGAGGTATGTCACGCAATGCCCCCAGC (OL-18) This sequence has been determined previously to be the optimal DNA recognition site of the AhR and ARNT complex (Swanson et al., 1995). The annealed nucleotides that were used as the competitor oligonucleotides for the EMSAs containing the mutated DRE (underlined) are 5′-TCGAGCTGGGGGCATTGATTGACATAC (HIS 108) and 3′-TCGAGGTATGTCAATCAATGCCCCCAGC (HIS 109).

Materials. The plasmid pLUC1A1, used to generate the stably transfected HepG2 cell line for the luciferase assays, was provided by Dr. Robert Tukey (University of California, San Diego). The HepG2-p450luc cell line was generated after transfection of HepG2 cells with the pLUC1A1 plasmid and clonal selection using neomycin. The LA-I cells, which lack expression of a functional AhR protein, were obtained from Dr. Jim Whitlock, Jr. (Stanford University, Stanford, CA). The anti-AHR immunoglobulins used for all experiments and the anti-ARNT immunoglobulins used for supershifting the EMSAs and Western blot analyses of ARNT were described previously (Pollenz, 1996). The anti-ARNT IgG used for Western blot analysis was provided by Dr. Gary Perdew (Penn State University, University Park, PA). The cytochrome P-450 1A1 antibody was purchased from XenoTech LLC (Kansas City, KS). The HSP90 antibody was purchased from Stressgen (Victoria, British Columbia, Canada). The actin antibody and the antirabbit IgG-linked agarose beads used for immunoprecipitation (IP) studies were purchased from Sigma (St. Louis, MO). The baculovirus-expressed ARNT was generated as described previously (Swanson et al., 1995).

Reagents. Tris-buffered saline/Tween 20 (TTBS) contains 50 mM Tris, 0.2% Tween 20, and 150 mM NaCl, pH 7.5. TTBS+ contains 50 mM Tris, 0.5% Tween 20, and 300 mM NaCl, pH 7.5. BLOTTO is 5% nonfat dry milk in TTBS. MENG is 25 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.5, 1 mM EDTA, 0.02% sodium azide, and 10% glycerol. Cracking buffer (2×) is 125 mM Tris, pH 6.8, 4% SDS, 25% glycerol, 4 mM EDTA, 0.05% bromphenol blue, 20 mM β-mercaptoethanol. Lysis buffer (2×) is 50 mM HEPES, pH 7.4, 40 mM sodium molybdate, 10 mM EGTA, 6 mM $MgCl_2$, 20% glycerol, and 1% Nonidet P-40. F buffer is 10 mM Tris, pH 7.05, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 μM $ZnCl_2$, 0.1 mM Na_3VO_4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml α_2-macroglobulin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 150 μM benzamidine, and 2.8 μg/ml aprotinin. The IP wash contains MENG, 150 mM NaCl, 1% Nonidet P-40, and 30 mM histidine. MDH is 3 mM $MgCl_2$, 25 mM HEPES, 1 mM dithiothreitol. TBE is 45 mM Tris base, 45 mM boric acid, 1 mM EDTA, pH 8.0.

Tissue Culture. HepG2 and Hepa-1 cells were grown until nearly confluent in Dulbecco's modified Eagle's medium and 8% fetal bovine serum. The cells were treated with Na_2MoO_4 or Na_2SO_4 (to control for the sodium content) for 12 h, followed by treatment with agonist [either 1 nM TCDD or 10 μM β-naphthoflavone (BNF)] for 1 h unless otherwise noted. The cells were harvested after two washes with ice-cold PBS, and the protein concentrations were determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL). To address the issue of cell viability in the presence of molybdate, we performed trypan blue staining of Hepa-1 cells that were cultured in the presence of increasing concentrations of sodium molybdate for 12 h. Viability was significantly affected (an 8% decrease in viability) at only the highest concentration (50 mM) of molybdate tested (data not shown). At the 35-mM concentration of molybdate, the viability of Hepa-1 cells was not significantly different from the untreated cells (data not shown).

Preparation of Whole Cell Lysates. Hepa-1 and HepG2 cells were treated with Na_2MoO_4 or Na_2SO_4 as described above in *Tissue Culture* followed by treatment of 1 nM TCDD or 10 μM BNF (6 h for the whole cell lysate preparation). Whole cell lysates of HepG2 and Hepa-1 cells used for Western blot analysis of AhR, cytochrome P-450 1A1, and actin were carried out as described previously (Pollenz, 1996). The total cell lysates for the IP assay and sucrose density gradient analysis were also carried out as described previously (Sommer et al., 1998).

Western Blot Analysis. Whole-cell lysates of HepG2 and Hepa-1 cells were performed as described above. Fifteen micrograms of each sample was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis as described previously (Pollenz, 1996). The following antibodies were diluted in BLOTTO and used for immunostaining: anti-AhR (1 μg/ml), anti-cytochrome P-4501A1 (1:1,000), anti-ARNT (1:2,000), anti-HSP90 (1:500), and antiactin (1:500). The secondary antibody was linked to horseradish peroxidase (Pierce) (1:10,000) for visualization.

IP Analysis of Total Cell Lysates and Nuclear Extracts. Hepa-1 cells were incubated in the presence or absence of 35 mM sodium molybdate as described in *Tissue Culture*. The Hepa-1 cells used for nuclear extracts were additionally incubated in ^{35}S-labeled methionine and cysteine at a concentration of 30 μCi/ml for 18 h before harvesting. The cells were washed twice with PBS, scraped, and either resuspended into F buffer, containing either 20 mM sodium molybdate or 20 mM sodium sulfate for total cell lysates (Sommer et al., 1998), or resuspended into MDH buffer after a brief incubation in 10 mM HEPES for nuclear extracts (Denison and Deal, 1990). The 0.4 M KCl nuclear extracts were dialyzed at 4°C for 90 min in 0.1 M KCl/MENG buffer. The antibody-linked agarose beads were generated by incubating a 10-μL slurry of antirabbit IgG agarose beads (Sigma) with 2 μg of anti-AHR or nonspecific IgG in 50 μL of MENG for 1 h at 4°C and washed twice with MENG. Aliquots of the total cell lysate samples (250 μg) or radiolabeled nuclear extracts [35 μg, incubated with 35 μg of unlabeled and dialyzed LA-I nuclear extracts as described previously (Chen and Perdew, 1994)] were incubated with agarose beads linked to rabbit IgG and anti-AHR in 200 μL of MENG for 90 min at 4°C. After three 10-min washes with IP wash buffer, the samples were applied to an SDS-PAGE gel. AhR, ARNT, and HSP90 proteins were visualized using Western blot analysis. The ^{35}S-labeled AhR protein was detected after PhosphorImager analysis.

Immunocytochemistry. Hepa-1 cells were grown on glass coverslips and then incubated in the absence or presence of 35 mM sodium molybdate or sodium sulfate for 12 h followed by treatment with 1 nM TCDD for 1 to 6 h. Immunofluorescence staining and microscopy was performed as described previously (Pollenz et al., 1994, 1996).

EMSA Analysis of Nuclear Extracts and Cytosolic Extracts. HepG2 and Hepa-1 cells were grown until nearly confluent, incubated in the absence or presence of Na_2MoO_4 or Na_2SO_4, and treated with either agonist or the vehicle control for 1 h. The cells were harvested and either cytosolic (Yang and DeFranco, 1996) or nuclear extracts (Denison and Deal, 1990) were prepared as described previously. Cytosolic extracts were harvested in MENG that contained either 20 mM Na_2MoO_4 or Na_2SO_4. Where indicated, the nuclear extracts (6 μg) were incubated in the absence or presence of either Na_2MoO_4 or Na_2SO_4 for 15 min at room temperature. Aliquots (6 μg) of the nuclear extracts were incubated with 7 μg of salmon testes DNA and KCl (final concentration, 0.1 M) in MENG buffer at room temperature for 10 min. Aliquots of the cytosolic extracts (50 μg) were incubated with 10 μM BNF in dimethyl sulfoxide (DMSO) or DMSO alone for 90 min at 30°C followed by incubation with KCl (final concentration, 50 mM) and 2 μg of salmon testes DNA for 10 min at room temperature. The samples that contained either the cytosolic or nuclear extracts were then incubated with ^{32}P-labeled OL 17/18 for an additional 10 min at room temperature. The samples were applied to a nondenaturing 4% polyacrylamide gel using 0.5× TBE as the running buffer. The results were quantified using PhosphorImager analysis.

Luciferase Assay. Nearly confluent HepG2-P-450luc cells were treated with Na_2MoO_4 and 10 μM BNF in DMSO or DMSO alone for 12 h. The cells were washed twice with PBS and harvested. The cells were then lysed by three cycles of freeze/thawing. The samples were centrifuged for 10 min at 8000 rpm at 4°C and the supernatants were analyzed by luciferase assay. Luciferase analysis was performed as outlined by Promega (Madison, WI). Results were normalized by protein concentration as determined by the bicinchoninic acid protein assay (Pierce).

Sucrose Density Gradient Analysis of Total Cell Lysates. Total cell lysates were isolated from Hepa-1 cells as described above in *IP Analysis of Total Cell Lysates*. Cell lysates (400 μg) were layered on 5.1-ml, 10 to 30% sucrose gradients in MENG buffer. The sealed centrifuge tubes were centrifuged in a Beckman Vti65.2 rotor at 416,000*g* for 3 h at 4°C. Fractions (200 μL) were collected and aliquots of each fraction were subjected to SDS-PAGE and transferred to a nitrocellulose membrane for Western blotting detection of the AhR as described above.

Results

Molybdate Stabilizes the HSP90-Associated Form of the AhR But Inhibits Formation of the AhR/ARNT Complex In Vivo. Our goal was to use sodium molybdate, an agent that stabilizes the association of HSP90 with a number of steroid receptors, as a tool to elucidate the role of HSP90 dissociation in the AhR signaling pathway. To ensure that molybdate stabilizes the AhR/HSP90 complex in vivo and to determine whether molybdate inhibits formation of the AhR/ARNT complex in the presence of agonist, we treated Hepa-1 cells with 35 mM sodium molybdate for 12 h before the administration of agonist (Fig. 1). In the presence of agonist, both 6S and 9S complexes could be detected in the whole-cell lysates (Fig. 1, B and E). Previous reports have identified the 9S complex of the AhR as that associated with HSP90, whereas the 6S form represents the AhR/ARNT heterodimer (Wilhelmsson et al., 1990; Perdew, 1991; Whitelaw et al., 1993). However, incubation of Hepa-1 cells with sodium molybdate before the addition of agonist eliminated the detection of the 6S peak, but had no significant effect on the 9S peak (Fig. 1, C and F). These data confirm that sodium molybdate stabilizes the 9S AhR/HSP90 form of the AhR and may inhibit formation of the AhR/ARNT complex in vivo.

Molybdate Inhibits the Induction of Cytochrome P-4501A1 Levels and Agonist-Induced Depletion of the AhR. Once we had determined that sodium molybdate stabilizes the AhR/HSP90 complex in vivo in the presence of agonist, we questioned whether molybdate might inhibit the AhR signaling pathway. Toward this end, we examined first the effects of sodium molybdate on two events that are mediated by agonist binding of the AhR: induction of cytochrome P-4501A1 levels and degradation of AhR protein. As shown in Fig. 2, treatment of either HepG2 or Hepa-1 cells with BNF significantly induced cytochrome P-4501A1 levels (lanes 1 and 2). The addition of sodium molybdate, however, significantly inhibited BNF induction of the cytochrome P-4501A1 levels in both HepG2 and Hepa-1 cells in a concentration-dependent manner (Fig. 2, A and B). In contrast, sodium sulfate, which was used as a control for the sodium component of sodium molybdate, did not affect agonist induction of cytochrome P-4501A1, indicating that the inhibition was specific to the presence of molybdate (data not shown). Molyb-

date inhibited agonist activation of the AhR signaling pathway in a concentration-dependent manner when either TCDD or BNF was used as agonist (data not shown). Therefore, molybdate's effects on the AhR pathway do not seem to be specific to a certain agonist, because TCDD and BNF differ greatly in chemical structure and metabolic half-life.

Although molybdate has been demonstrated previously to exert species-specific effects on the stabilization of the AhR in vitro, we did not observe a difference in CYP1A1 induction when treating either the HepG2 cells or the Hepa-1 cells with 10 μM BNF, although we saw a slight difference with the use of 1 nM TCDD (data not shown). Previous data has demonstrated that although the presence of molybdate in buffers used to isolate the AhR from human sources increases detection of the human AhR using radioligand-based approaches, molybdate has little effect on detection of the rat and murine AhR (Denison et al., 1986; Roberts et al., 1990; Henry and Gasiewicz, 1993). It has been hypothesized that the failure of molybdate to exert an observable effect on the AhR-HSP90 complex in these studies may because the AhR isolated from the C57 black mouse is more tightly associated with HSP90 compared with that isolated from other sources (Cuthill et al., 1987; Roberts et al., 1990; Dolwick et al., 1993a).

Previous studies have established that the AhR is rapidly degraded in vivo after ligand binding (Pollenz, 1996; Giannone et al., 1998; Roman et al., 1998). In addition, the degradation of the AhR seems to require disruption of the AhR-HSP90 complex (Chen et al., 1997). To determine whether stabilization of the AhR-HSP90 complex with molybdate would inhibit the agonist-induced degradation of the AhR, either HepG2 or Hepa-1 cells were treated with sodium molybdate for 12 h before the administration of TCDD for 6 h (Fig. 3). In the absence of molybdate, TCDD treatment of Hepa-1 cells resulted in approximately >80% loss of AhR protein. However, this TCDD-induced depletion was blocked in a concentration-dependent manner by incubation of the cells with molybdate, before the administration of agonist. Molybdate similarly inhibited the TCDD-induced degradation of the AhR protein in HepG2 cells (Fig. 3B). Interestingly, the treatment of molybdate alone (20 mM) reduced

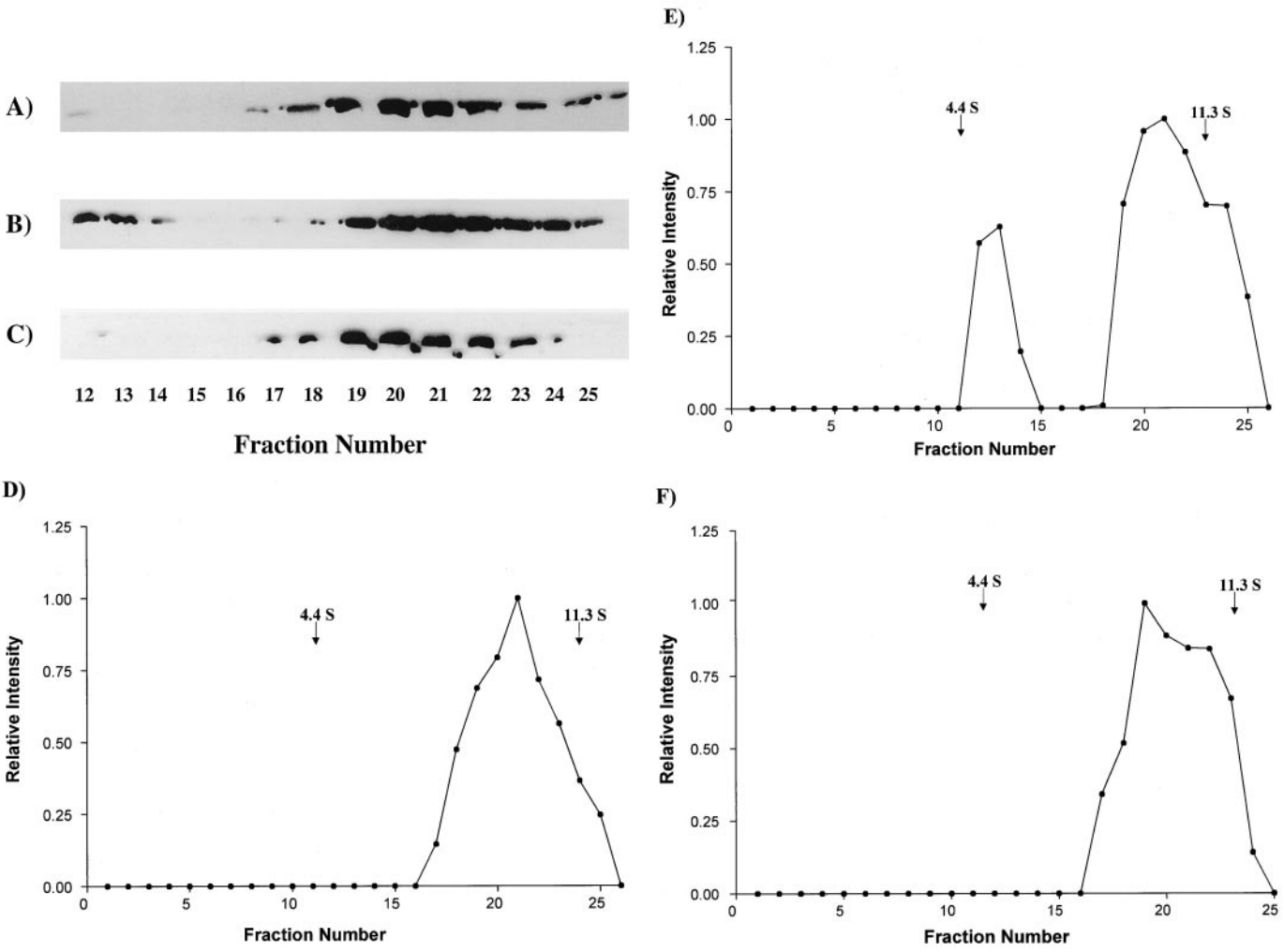

Fig. 1. Sodium molybdate stabilizes the HSP90-associated form of the AhR in the presence of agonist in vivo. Hepa-1 cells were treated in the absence or presence of 35 mM sodium molybdate for 12 h, after which 10 μM BNF or DMSO (vehicle control) was added for 1 h. Cells were harvested and whole cell lysates were performed as described under *Experimental Procedures*. Total cell lysates (400 μg) were applied to a 10 to 30% sucrose density gradient and the amount of AhR in each fraction was determined by Western blot analysis using anti-AhR immunoglobulins as described under *Experimental Procedures*. Samples are as follows: A, DMSO only. B, BNF only. C, BNF and molybdate pretreated. AhR amounts in each fraction were quantitated by densitometry analysis, samples as follows: D, DMSO only; E, BNF only; F, BNF and molybdate pretreated. The protein markers bovine serum albumin (4.4 S) and catalase (11.3 S) were used as external standards.

AhR protein levels in Hepa-1 cells to approximately 75% of control; however, the administration of molybdate alone did not affect AhR levels in HepG2 cells. Because the presence of molybdate inhibited agonist-induced degradation of the AhR, these data further imply that molybdate stabilizes the interaction between the AhR and HSP90 in both HepG2 and Hepa-1 cells.

Molybdate Inhibits *AhR* Gene Activation in a Concentration-Dependent Manner. Because molybdate inhibits the agonist induction of both cytochrome P-4501A1 and AhR degradation, we questioned whether these effects may be caused by a direct inhibition of the ability of the AhR/ARNT complex to activate gene transcription. For these studies, a stably transfected HepG2 cell line containing the promoter region of cytochrome P-4501A1 upstream of a luciferase reporter gene (HepG2-P-450luc) was used to assay AhR/ARNT gene transcription. Administration of 20 mM molybdate inhibited BNF induction of luciferase activity by 75%, whereas a 50 mM concentration of molybdate resulted in a 95% inhibition of luciferase activity (Fig. 4).

Molybdate Decreases the Nuclear AhR/ARNT Levels Formed in Vivo. Once we had determined that molybdate inhibits AhR-mediated gene transcription in a concentration-dependent manner, we questioned whether this inhibition was caused by an absence of the nuclear AhR/ARNT heterodimer. To address this question, HepG2 cells were treated with varying concentrations of either sodium sulfate or sodium molybdate before the addition of agonist, and nuclear extracts were prepared and analyzed by EMSA. First, we verified that the observed complex obtained from the BNF-treated nuclear extracts was composed of the AhR and ARNT proteins as treatment with BNF but not DMSO induced the formation of a single complex (Fig. 5A, lanes 1 and 2). The absence of the DNA binding complex after the addition of either the AhR or ARNT antibodies (Fig. 5A, lanes 3–4), but not the nonspecific IgG (Fig. 5A, lane 5), confirms that the complex is composed of both the AhR and ARNT.

Nuclear extracts prepared from HepG2 cells treated with sodium molybdate for 12 h, followed by BNF treatment for 1 h, reveal a concentration-dependent decrease in the formation of the AhR/ARNT DNA binding complex (Fig. 5B, lanes 7–11; Fig. 5C). To verify that these results were specific for the molybdate anion, similar experiments were performed using sodium sulfate, to control for the sodium component of sodium molybdate. As shown in Fig. 5, B (lanes 1–5) and C, only the highest concentration of sodium sulfate affected DNA binding of the AhR/ARNT complex in vivo, yet the samples treated with sodium molybdate at the same dose were significantly lower than those treated with sodium sulfate (78% and 32% decreases, respectively). Treatment of 20 mM sodium molybdate resulted in a 63% decrease in complex formation, whereas treatment of the same concentration of sodium sulfate had no significant effect. Similar results were obtained after analysis of nuclear extracts prepared from Hepa-1 cells (data not shown).

Molybdate Does Not Inhibit the Ability of the Preformed AhR/ARNT Complex to Interact with DNA. Because the administration of molybdate resulted in decreased AhR/ARNT complex formation from nuclear extracts, we questioned whether this effect might be caused by direct inhibition of DNA binding of the AhR/ARNT heterodimer. To address this question, we prepared nuclear extracts from cells treated with BNF and incubated the BNF-induced extracts with varying concentrations of either sodium molybdate or sodium sulfate (Fig. 6). Previous results have shown that the presence of molybdate in buffers used to prepare nuclear extracts from cells pretreated with agonist does not affect the formation of the AhR/ARNT complex (Roberts et al., 1990). Relatively low concentrations (0.25–50 mM) of either sodium molybdate or sodium sulfate were found to increase formation of the AhR/ARNT DNA binding complex (Fig. 6A), probably because of the increased sodium concentrations present in the assay. The observation that the levels of the AhR/ARNT DNA binding complex formed in the pres-

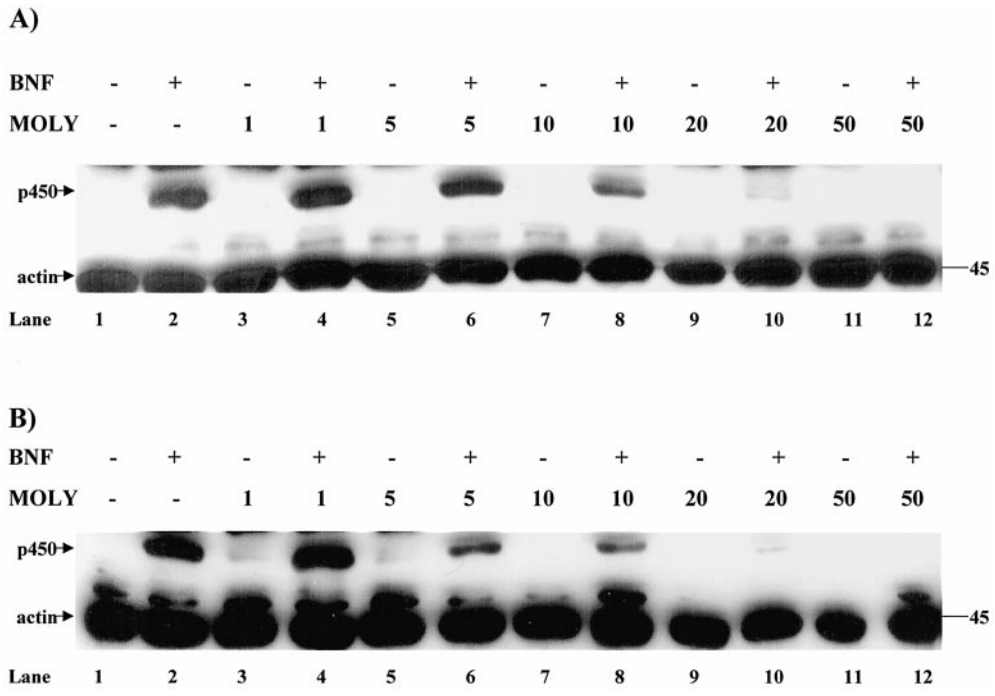

Fig. 2. Inhibition of agonist-induced cytochrome P-4501A1 protein levels by molybdate. HepG2 (A) and Hepa-1 cells (B) were treated with the indicated concentrations of sodium molybdate for 12 h, treated with either 10 μM BNF or DMSO for 6 h, and harvested. Whole cell lysates were prepared as described under *Experimental Procedures*. Aliquots (15 μg) were applied to an 8% SDS-PAGE gel and subjected to Western blot analysis using anti-cytochrome P-450 1A1 and anti–β-actin immunoglobulins. The molecular mass marker of 45 kDa is indicated at the right of the gel.

ence of sodium sulfate were not significantly different from those formed in the presence of sodium molybdate indicates that sodium molybdate does not inhibit DNA binding of the preformed AhR/ARNT heterocomplex in vitro at the 20 and 50 mM concentrations of sodium molybdate that inhibited AhR/ARNT function in vivo (Fig. 6B).

Immunofluorescence Microscopy of Hepa-1 Cells Indicates That Molybdate Does Not Inhibit Nuclear Translocation of the AhR and Has No Effect On ARNT Localization. We next questioned whether the molybdate-induced decrease in active AhR/ARNT complex as depicted in Fig. 5 might reflect an ability of molybdate to stabilize the AhR-HSP90 complex such that the AhR is incapable of entering the nucleus and interacting with ARNT. To test this idea, we examined the effect of molybdate on nuclear translocation of the AhR. Hepa-1 cells were grown on glass coverslips, preincubated with 35 mM molybdate for 12 h, and then stimulated with TCDD (1 nM) for either 1 or 6 h. After each treatment, the cells were fixed and stained for AhR as detailed previously (Pollenz et al., 1994, 1996). A representative experiment is shown in Fig. 7. Control cells treated with 35 mM sodium sulfate showed cytoplasmic staining that became predominately nuclear after 1 h of TCDD treatment and then decreased to near background levels after 6 h of TCDD treatment (Fig. 7, A–C). These results are consistent with the TCDD-induced nuclear translocation and subsequent degradation of the AhR (Pollenz et al., 1994, 1996). When Hepa-1 cells were incubated with 35 mM molybdate, the cell morphology became slightly altered, but the AhR staining pattern remained predominantly cytoplasmic and of similar intensity to control-treated cells (Fig. 7, D and H). Treatment of cells with molybdate prior to treatment with TCDD for 1 h resulted in reduced AhR staining in the cytoplasm and a concomitant increase in AhR staining in the nucleus (Fig. 7, E–G). The redistribution of staining was observed independent of the morphology of the cell (compare Fig. 7E with Fig. 7G). Importantly, the treatment of molybdate before the administration of TCDD for 6 h did not result in reduced levels of AhR staining in the nucleus. Instead, the viable cells all showed intense staining within the nuclear compartment that was of similar intensity to that of cells treated with TCDD for 1 h (Fig. 7, I–L). This finding is consistent with the Western blot analysis of AhR that showed molybdate blocked TCDD-induced AhR degradation (Fig. 3). In addition, these results strongly indicate that the AhR-HSP90 complex is capable of entering the nucleus after ligand binding. This finding is particularly relevant because the ARNT protein was also localized to the nucleus of molybdate-treated cells (Fig. 7N). Thus, despite the fact that AhR and ARNT are present in the same subcellular compartment, they seem to be incapable of interacting to form an active AhR/ARNT heterodimer when molybdate is present.

Molybdate Inhibits the In Vitro Formation of the AhR/ARNT Complex. Once we had determined that molybdate did not inhibit AhR nuclear translocation, we questioned whether molybdate inhibits the ability of the AhR to dimerize with ARNT and in this manner diminishes the amount of active AhR/ARNT complex found in the nucleus. To address this issue, we prepared cytosolic extracts from Hepa-1 cells cultured in the absence or presence of molybdate, initiated formation of the AhR/ARNT DNA binding complex with the addition of agonist and performed EMSAs. The addition of BNF to cytosolic extracts prepared from cells incubated in the absence of molybdate significantly induced

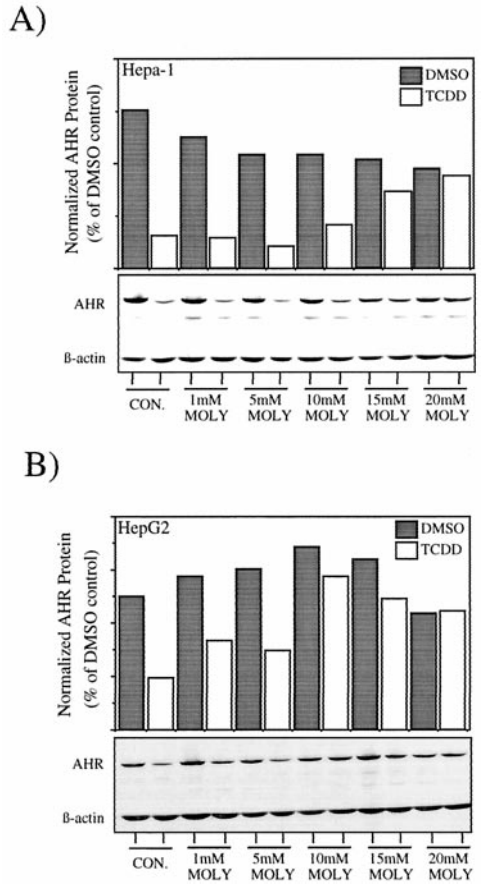

Fig. 3. Molybdate inhibition of agonist-induced depletion of AhR. Hepa-1 (A) and HepG2 cells (B) were treated with sodium molybdate for 12 h, followed by treatment with either 1 nM TCDD or DMSO for 6 h and harvesting. Aliquots (15 μg) of the whole cell lysates were applied to an 8% SDS PAGE gel and subjected to Western blot analysis using anti-AHR and anti–β-actin immunoglobulins as described under *Experimental Procedures*.

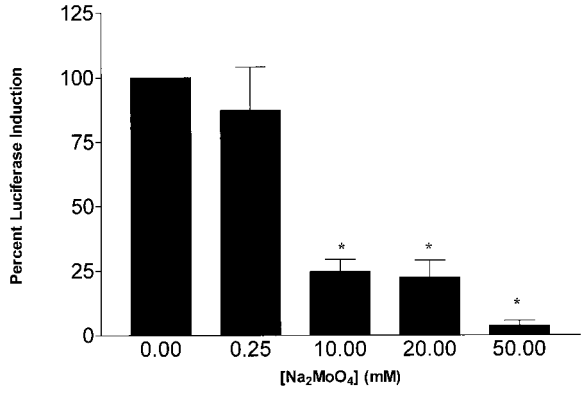

Fig. 4. Molybdate inhibits AhR-mediated gene activation in a concentration-dependent manner. The HepG2-p450luc cells were treated with the indicated concentrations of sodium molybdate and either 10 μM BNF or the vehicle control, DMSO, for 12 h. The cells were harvested and the luciferase assays were performed as described under *Experimental Procedures*. Luciferase induction was normalized to the BNF only samples and was normalized to protein concentration. The data represents average values ± S.E.M. obtained from three separate experiments. *$P < .001$ (different from the BNF-only samples using one-way ANOVA).

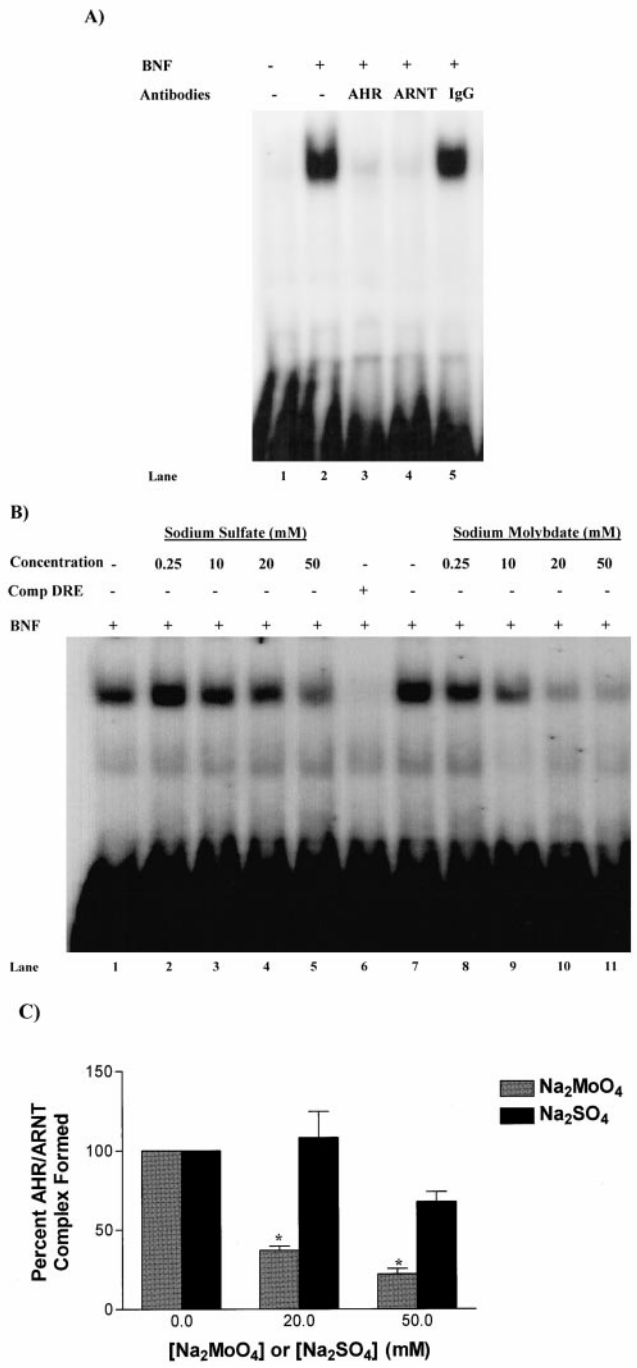

Fig. 5. Sodium molybdate inhibits the in vivo formation of the AhR/ARNT DNA binding complex. A, characterization of the AhR/ARNT DNA binding complex obtained from HepG2 cells. HepG2 cells were treated with either vehicle control (lane 1) or 10 μM BNF (lanes 2–5) for 1 h before harvesting. The nuclear extracts were isolated as described under *Experimental Procedures* and analyzed by EMSA in the presence of 0.6 μg anti-AHR (lane 3), 0.4 μg anti-ARNT (lane 4), or 0.6 μg nonspecific IgG (lane 5). B, sodium molybdate inhibits the in vivo formation of the AhR/ARNT DNA binding complex in HepG2 cells. HepG2 cells were treated with either sodium sulfate (lanes 2–5) or sodium molybdate (lanes 8–11) for 12 h, followed by 10 μM BNF for an additional hour, and nuclear extracts were prepared. Aliquots (6 μg) were subjected to EMSA. Lane 6 represents samples that were incubated with an excess of the oligonucleotide HIS 17/18 (DRE) to demonstrate complex specificity. C, the AhR/ARNT DNA binding complex depicted in part B was quantified by PhosphorImager analysis. The data was normalized to the BNF-only samples (lane 1) and is the result of three separate experiments. *$P <$ .001 (significantly different from the BNF-treated samples and from sodium sulfate-treated samples using two-way ANOVA).

formation of the AhR/ARNT DNA binding complex by 2.5 fold when compared with that treated with DMSO ($P <$ .001) (Fig. 8A, lanes 1–2, and Fig. 8B). However, when similar experiments were performed using the cytosolic extracts prepared from cells incubated with molybdate, BNF did not induce significant formation of the AhR/ARNT binding complex (Fig. 8A, lanes 3–4, and Fig. 8B). To verify that the observed DNA binding complex represented the AhR/ARNT complex, we performed competition experiments using either oligonucleotides that contained either the excess wild-type DRE (lane 5) or mutated DRE (lane 6). Thus, because molybdate did not inhibit DNA binding of the preformed AhR/ARNT complex (Fig. 6), but inhibited the in vitro activation of the AhR/ARNT DNA binding complex (Fig. 8), we reasoned that it may act by inhibiting the ability of the AhR to interact with ARNT.

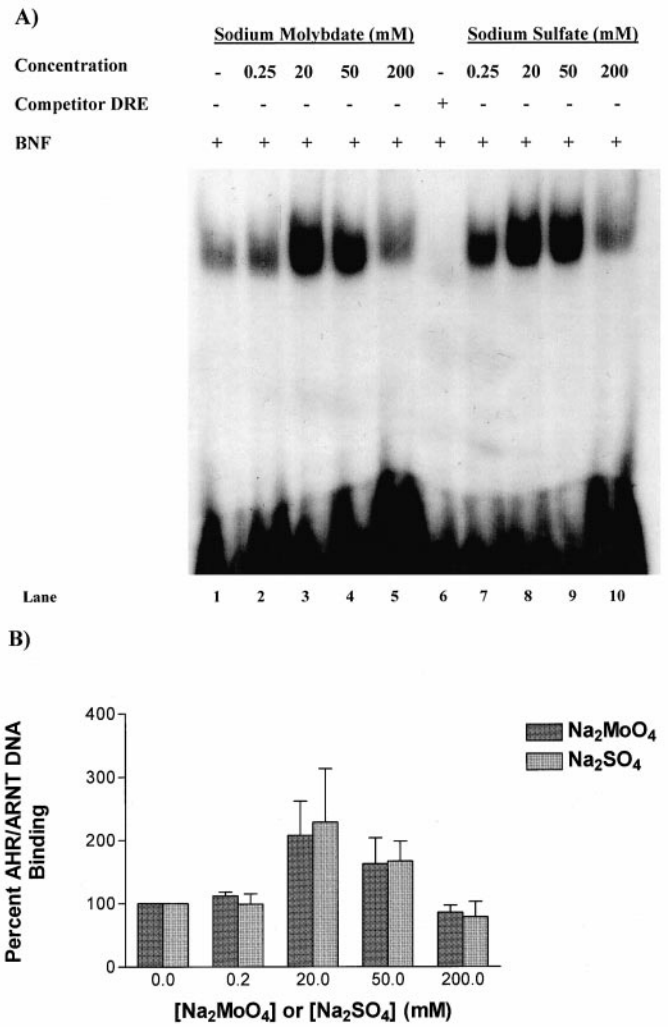

Fig. 6. Sodium molybdate does not inhibit the DNA binding of the preformed AhR/ARNT complex. A, HepG2 cells were treated with 10 μM BNF for 1 h and nuclear extracts were prepared. Aliquots (6 μg) were incubated with either sodium molybdate (lanes 2–5) or sodium sulfate (lanes 7–10) for 15 min at room temperature before being subjected to EMSA. B, the AhR/ARNT DNA binding complex depicted in part A was quantified by PhosphorImager analysis. The data was normalized to the BNF-treated sample (lane 1) and is the result of three separate experiments. The sodium molybdate- and sodium sulfate-treated samples were not found to be statistically significant from one another using two-way ANOVA.

Molybdate Inhibits Dimerization of AhR with ARNT, But Stabilizes the Association of AhR with HSP90 in the Nucleus. To verify that molybdate inhibits the formation of the AhR/ARNT heterodimer and stabilizes the AhR/HSP90 complex in vivo, we performed IP experiments with an antibody specific for AhR. Either whole-cell lysates or nuclear extracts were prepared from Hepa-1 cells that had been pretreated with molybdate for 12 h followed by BNF treatment for 1 h before harvesting. The AhR-associated proteins were precipitated from either the whole cell or nuclear extracts and analyzed by Western blot analysis (Fig. 9).

To determine whether molybdate inhibits the ability of the AhR to dimerize with ARNT, the AhR was immunoprecipitated from whole cell extracts and the immunoprecipitates were analyzed by Western blot analysis using the ARNT antibody. The following indicates that the detection of ARNT shown in Fig. 9A is specific for the agonist induction of AhR-associated proteins: 1) ARNT was not detected when the total cell lysates prepared from the BNF-treated cells were incubated with nonspecific IgG (Fig. 9A, lane 2) and 2) ARNT was not detected when the total cell lysates were prepared from the vehicle control (DMSO-treated cells) (Fig. 9A, lanes 3 and 4). The addition of molybdate to the cells significantly inhibited the BNF-induced formation of the AhR/ARNT complex (Fig. 9A, lane 6) compared with BNF induction alone (Fig. 9A, lane 5). To verify that relatively equal amounts of the AhR were precipitated in these samples, aliquots were analyzed for the detection of the AhR using Western blot analysis (Fig. 9B).

To determine whether molybdate enhances the amount of AhR that is associated with HSP90 in the presence of agonist, we immunoprecipitated the AhR from ^{35}S-labeled nuclear extracts and performed either Western blot analysis using the HSP90 antibody (Fig. 9C) or PhosphorImager analysis of the ^{35}S-labeled AhR band to normalize the amount of AhR precipitated (Fig. 9D). The specificity of the antibody interactions is indicated by the presence of the HSP90 band in the immunoprecipitates that contained the AhR antibody (Fig. 9C, duplicate lanes 3, 4, and lane 6) but not in those that contained the nonspecific IgG antibody (Fig. 9C, lanes 1 and 2). In addition, the AhR-HSP90 complex was detected in the nuclear extracts only in the presence of agonist (Fig. 9C, lanes 3–6). These results confirm previous reports that an HSP90-associated form of the AhR can be observed in the nucleus of agonist-treated cells (Wilhelmsson et al., 1990; Perdew, 1991). Finally, the presence of molybdate increases the amount of agonist-induced nuclear AhR associated with HSP90 by approximately 3-fold compared with that of the agonist alone (Fig. 9C, lanes 3, 4, and 6). This increase over agonist alone is similar to the effects of molybdate on stabilizing the HSP90/GR complex (Yang and DeFranco, 1996). These data also indicate that molybdate inhibits the ability of the AhR to dimerize with ARNT by inhibiting its ability to dissociate from HSP90.

Discussion

In this study, we have employed sodium molybdate to stabilize the HSP90-associated form of the AhR and to test the idea that a primary role of HSP90 association with the AhR is to inhibit nuclear translocation of the AhR. Although molybdate stabilized the 9S form of the AhR (Fig. 1) and inhibited its signaling pathway (Figs. 2–4), it did not inhibit nuclear translocation of the AhR (Fig. 7). Although it is possible that molybdate may adversely affect the post-translational folding/modification of the AhR, this is unlikely be-

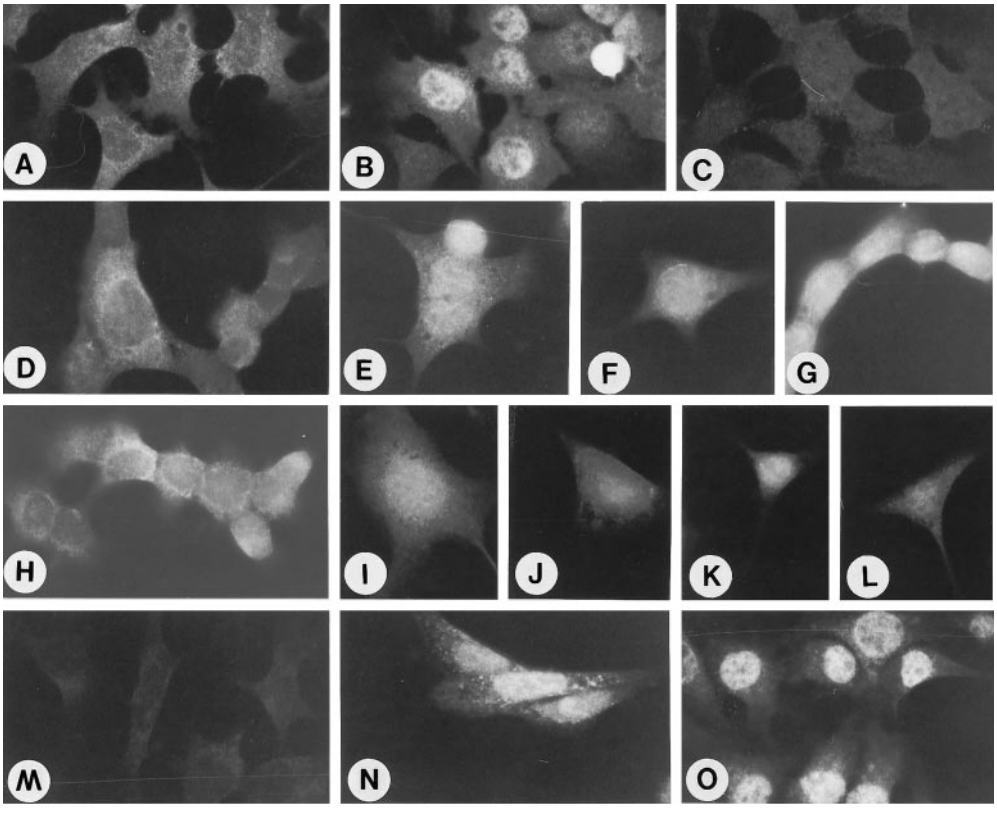

Fig. 7. Immunofluorescence microscopy of Hepa-1 cells indicates that molybdate does not inhibit nuclear translocation of the AhR and has no effect on ARNT levels. Hepa-1 cells were grown on glass coverslips and treated with sodium molybdate or sodium sulfate for 12 h, followed by treatment of 1 nM TCDD for 1 to 6 h and staining with anti-AHR or anti-ARNT as outlined under *Experimental Procedures*. Samples were stained with anti-AHR unless noted. Description of panels: A, DMSO, 35 mM sodium sulfate; B, 1 nM TCDD, 1 h, 35 mM sodium sulfate; C, 1 nM TCDD, 6 h, 35 mM sodium sulfate; D and H, DMSO, 35 mM sodium molybdate; E to G, 1 nM TCDD, 1 h, 35 mM sodium molybdate; I to L, 1 nM TCDD, 6 h, 35 mM sodium molybdate; M, DMSO, nonspecific IgG; N, DMSO, 35 mM sodium molybdate, anti-ARNT; O, DMSO, anti-ARNT.

cause agonist induction of AhR nuclear transport remains intact in the presence of molybdate. Thus, we next pursued the idea that by stabilizing the association between the AhR and HSP90, molybdate inhibits AhR/ARNT dimerization (Figs. 8 and 9).

Although the AhR is a basic helix-loop-helix protein, the mechanisms that regulate its signaling pathway have long been compared with that of the steroid receptors, in particular, that of the GR. A common hypothesis is that dissociation of the HSP90 complex from either of these receptors is initiated by agonist binding, resulting in an unmasking of their nuclear translocation signals and allowing nuclear transport of the receptors (Yang and DeFranco, 1996; Pratt and Toft, 1997). Once in the nucleus, the GR homodimerizes, whereas the AhR heterodimerizes with ARNT. However, studies of both the AhR and GR have challenged this model. First, previous work using cell fractionation techniques have shown that both HSP90-associated and HSP90-free forms of the AhR may be recovered from the nucleus of cells treated with agonist (Wilhelmsson et al., 1990; Perdew, 1991). However, this work did not directly examine the role of HSP90 in nuclear translocation of the AhR or closely examine the molecular events in the nucleus that occur after HSP90 dissociation from the AhR and AhR/ARNT dimerization. Second, the AhR has been localized to the nucleus of a number of cell types in the absence of ligand (R. Pollenz, unpublished results). Third, similar studies examining the GR have shown that both the unliganded HSP90-associated GR and liganded HSP90-free GR are capable of accessing the nucleus (Hache et al., 1999). Finally, as we have demonstrated in this study, the HSP90-associated AhR that is stabilized by molybdate is capable of traversing the nuclear membrane in the presence of its agonists, BNF and TCDD (Fig. 7 and 9). Together, these studies imply that agonists promote retention of the receptors in the nucleus by undetermined yet dynamic means that do not involve dissociation of HSP90 and support the hypothesis that HSP90 may play a role in cytoplasmic/nuclear trafficking.

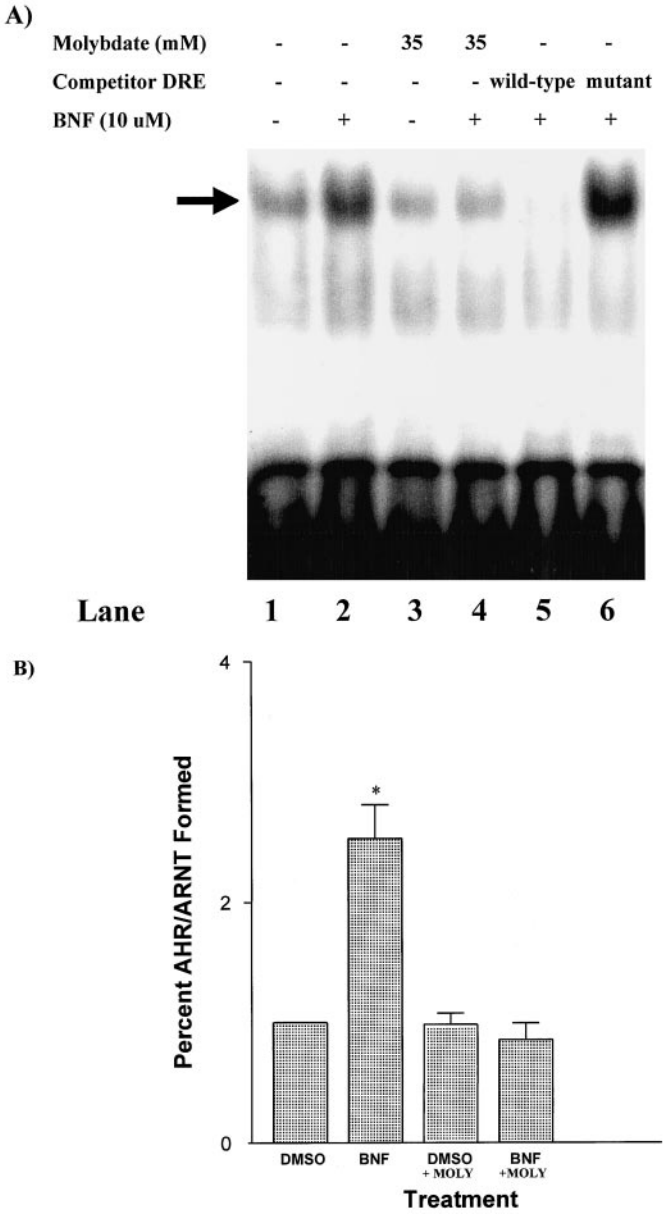

Fig. 8. Molybdate inhibits the in vitro activation of the AhR/ARNT DNA binding complex. A, Hepa-1 cells were incubated in the absence or presence of molybdate (35 mM) for 12 h and cytosolic extracts were prepared as described under *Experimental Procedures*. Aliquots of the extracts (50 μg) were incubated with either 10 μM BNF or the vehicle control for 90 min at 30°C. The samples were incubated with ^{32}P-labeled OL 17/18, and applied to a 4% nondenaturing poly-acrylamide gels, followed by PhosphorImager analysis. Wild-type DRE is the OL 17/18 oligonucleotide, and mutant DRE is the HIS 108/109 oligonucleotide. The arrow indicates the AhR/ARNT DNA binding complex. B, the AhR/ARNT DNA binding complex depicted in A was quantified by PhosphorImager analysis. The data was normalized to the DMSO-only treated samples (lane 1) and is the result of four separate experiments. *$P < .001$ (significantly different from the DMSO-treated sample using one-way ANOVA.

Within the PAS domain, the AhR/HSP90 interaction colocalizes with the ligand-binding region and the surface that interacts with ARNT (Fukunaga et al., 1995; Whitelaw et al., 1995; Perdew and Bradfield, 1996), whereas within the basic region, it colocalizes with both the DNA binding region (Fukunaga et al., 1995; Perdew and Bradfield, 1996) and the nuclear localization sequence (Ikuta et al., 1998). The basic region of the AhR is believed to be associated with only one molecule of HSP90, whereas the PAS region seems to be associated with both molecules of HSP90 (Fukunaga et al., 1995). We propose that the binding of ligand to the AhR could alter the conformation of the AhR such that the interaction of HSP90 with the basic region is disrupted, allowing the characterized NLS to be exposed and permitting import of the AhR-HSP90 complex into the nucleus. The stabilization of the AhR-HSP90 complex by molybdate may only affect the stronger interactions with the PAS domain of the AhR and may not interfere with potential ligand-induced changes in the interplay between HSP90 and the basic region of the AhR.

The association of HSP90 with the PAS domain of the AhR seems to inhibit its ability to dimerize with ARNT. Deletion of the PAS domain of the AhR, the region that interacts strongly with HSP90, has been shown to result in a form of the AhR that interacts with ARNT in a ligand-independent manner in vitro (Dolwick et al., 1993b; Antonsson et al., 1995). In this study, we demonstrated that stabilization of the HSP90-associated form of the AhR inhibits its dimerization with ARNT (Fig. 9) and further supports the hypothesis that the association of HSP90 with the PAS domain sterically hinders the ability of the AhR to interact with ARNT. The observation that a ligand-bound form of the AhR that re-

mains associated with HSP90 fails to interact with ARNT implies that some key regulatory step such as phosphorylation after the nuclear import of the AhR-chaperone complex must occur to allow the AhR to dissociate from HSP90 and dimerize with ARNT. Therefore, AhR/ARNT dimerization seems to be a regulated process that occurs in the nucleus.

In contrast to the interaction of HSP90 with the PAS domain of the AhR, the interaction with the basic region is thought to enhance DNA binding of the AhR/ARNT complex. Crystallographic studies of the DNA binding forms of basic helix-loop-helix proteins have shown that as the basic regions of these proteins contact DNA, they undergo a transition from a random coil to an α helix (Ferré-D'Amaré et al., 1994). It has been proposed previously that the interaction of HSP90 at the basic region of the AhR may assist in DNA binding of the AhR/ARNT heterodimer by facilitating this transition (Antonsson et al., 1995). This idea is supported by data demonstrating that HSP90 aids in DNA binding of the basic helix-loop-helix heterodimers of MyoD/E12 and the MyoD and E12 homodimers after their oligomerization (Shue and Kohtz, 1994).

In this study, we have demonstrated that stabilization of the HSP90-associated form of the AhR inhibits two disparate events that are triggered by the addition of agonist, activation of gene transcription, and degradation of the AhR (Figs. 1–4). Agonist-induced degradation of the AhR does not seem to involve gene transcription; rather, it is probably a regulated proteolytic event (Giannone et al., 1998; Roman et al., 1998). The observation that molybdate, which stabilizes HSP90 heterocomplexes, protected the AhR from degradation (Fig. 3), whereas geldanamycin, which disrupts HSP90 heterocomplexes, enhanced degradation of the AhR (Chen et al., 1997) suggests that dissociation of HSP90 is a critical step in this pathway. We propose that HSP90 may affect the molecular events involved in the cytoplasmic/nuclear trafficking as well as activation of the AhR in the nucleus by mediating AhR/ARNT dimerization and subsequent AhR/ARNT DNA binding. Like the GR, the AhR may shuttle

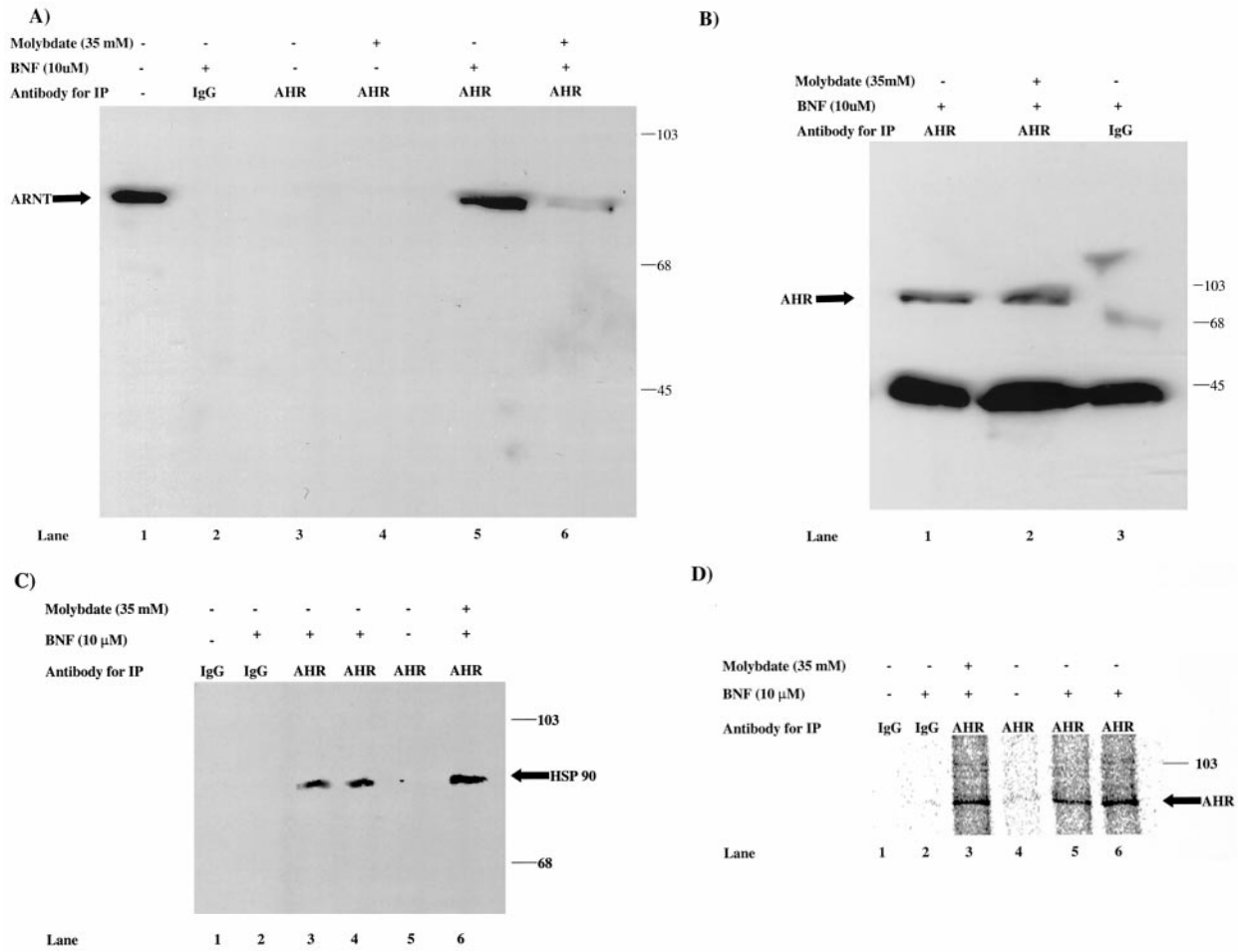

Fig. 9. Molybdate inhibits dimerization of the AhR with ARNT, but stabilizes the association of AhR with HSP90 in the nucleus. Protein-protein interactions of the AhR/ARNT and AhR/HSP90 complexes were analyzed using coimmunoprecipitation assays. Hepa-1 cells were incubated in the absence or presence of sodium molybdate for 12 h followed by administration of either 10 μM BNF or the vehicle control for 1 h. Whole cell lysates (A and B) or ^{35}S-labeled nuclear extracts (C and D) were prepared as described under *Experimental Procedures* and aliquots were incubated with agarose beads linked to rabbit IgG/anti-AHR for 90 min at 4°C. The samples were washed, applied to a 7.5% SDS-PAGE gel, and either transferred to a nitrocellulose membrane (A-C) or dried and visualized by PhosphorImager analysis (D). The membranes were stained with either (A) anti-ARNT (1:1000) (lane 1 in A consists of baculovirus-expressed ARNT that was used as a positive control), (B) anti-AHR (1 μg/μl), or (C) anti-HSP90 (1:500) immunoglobulins. D, PhosphorImager analysis of ^{35}S-labeled nuclear extracts immunoprecipitated with anti-AHR for normalization of labeled extract stained for anti-HSP90 in Fig. 9C (lanes 5 and 6 in D correlate to bands 3 and 4 in C, respectively). The molecular mass markers are indicated toward the left of the gels. The data is representative of two independent experiments.

between the cytosolic and nuclear compartments in its unliganded form. In summary, we have shown that 1) HSP90 dissociation is not required for nuclear translocation of the AhR, 2) HSP90 dissociation is essential for formation of the AhR/ARNT heterodimer, and 3) an additional undefined regulatory step is required for AhR/ARNT dimerization in the nucleus.

Note Added in Proof. Results published by Lees and Whitelaw while this manuscript was in review also confirm the ability of the AHR/HSP90 complex to translocate into the nucleus. Lees MJ and Whitelaw ML (1999) Multiple roles of ligand in transforming the dioxin receptor to an active basic helix-loop-helix PAS transcription factor complex with the nuclear protein Arnt. *Mol Cell Biol* **19:**5811–5822.

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