Inflammatory cytokine interleukin 1β induces inducible nitric-oxide synthase (iNOS) mRNA and its protein, which are followed by increasing the production of nitric oxide, in primary cultures of rat hepatocytes. Nuclear factor-κB (NF-κB), an important transcription factor for iNOS gene expression, is also activated and translocated to the nucleus. In the present study, we found that iNOS is present in tissues such as the endothelium and brain (2, 3), but iNOS is activated in organs including vascular vessels, kidney, and liver (4–6). Induction of iNOS by lipopolysaccharide contributes to the pathogenesis of septic shock (7), leading to organ destruction, including the liver. We (8) and others (9) have reported that a single cytokine interleukin 1β (IL-1β) mimicked the in vivo induction of iNOS in cultured hepatocytes. Other cytokines such as IL-6, tumor necrosis factor α (TNFα), and interferon γ had no effect under the same conditions (8), although a combination of these cytokines and lipopolysaccharide stimulated the nitric oxide production (10, 11). A high level of nitric oxide production by iNOS influences various hepatic metabolism and functions, where nitric oxide inhibits the mitochondrial Krebs cycle enzyme (12) and ATP synthase (13).

IL-1β is a key mediator in the inflammatory response and has been shown to activate the transcription factor, nuclear factor-κB (NF-κB), that is critical for the inducible expression of many genes involved in inflammation (14). The promoters of murine and human genes encoding iNOS contain a consensus sequence for the binding of NF-κB (15–17), which is necessary to confer inducibility by cytokines. To induce iNOS mRNA, activation of the transcription factor NF-κB is essential although not sufficient by itself (18–20). Nuclear translocation of NF-κB is triggered by changes in the redox state (21). As mentioned before, IL-1β activated and translocated NF-κB to the nucleus, resulting in the induction of iNOS gene expression, in primary cultures of rat hepatocytes (22). NF-κB is typically found in the form of p50/p65 heterodimers attached to the inhibitory molecule (IκB) in the cytoplasm of cells (23). Activation of NF-κB involves (i) proteolytic degradation of IκB following the phosphorylation by IκB kinase (24, 25), (ii) translocation of NF-κB to the nucleus, and (iii) binding to the promoter κB site of a target gene. The last step requires reduced cysteine residue(s) of NF-κB (26).

However, the role of the thiol residues in NF-κB molecule on the induction of iNOS in hepatocytes is obscure. deVeres et al. (27) reported that sodium arsenite inhibited cytokine-inducible nitric-oxide synthase expression in rat hepatocytes. Recently, Shumilla et al. (28) reported that thiol-reactive metals such as chromium, cadmium, mercury, zinc, and arsenite inhibited NF-κB binding to DNA in vitro. Phenylarsine oxide (PAO), arsenate derivative, interacts strongly with vicinal dithiol-containing molecules including enzymes and transcription factors (29). PAO also interacts with small molecular weight dithiol compounds such as 2,3-dimercaptopropanol and 1,4-dithiothreitol (DTT) but hardly interacts with monothiol compounds (30). These reports prompted us to investigate whether arsenate derivative binds to PAO, inhibits the production of iNOS by IL-1β in hepatocytes and, if so, which step of iNOS induction including NF-κB activation is influenced by PAO. In the present study, we found that PAO
inhibited IL-1β-induced nitric oxide production by blocking a step of NF-κB binding to DNA, but not the translocation of NF-κB into the nucleus.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human IL-1β (2 × 10^6 units/mg of protein) was generously provided by Otsuka Pharmaceutical Co. (Tokushima, Japan). [γ-32P]ATP (122 TBq/mmol) and [α-32P]dCTP (111 TBq/mmol) were from DuPont NEN Japan (Tokyo). PAO was purchased from Aldrich and dissolved in dimethyl sulfoxide. All other chemicals were of reagent grade.

**Cultures**—Hepatocytes were isolated from male Wistar strain rats (200–250 g) by collagenase perfusion as described previously (31). All animal experiments were approved by the Animal Care Committee of Kansai Medical University. The isolated hepatocytes were suspended in Williams’ medium E supplemented with 10% newborn calf serum, 100 units/ml penicillin (100 units/ml), streptomycin (0.1 mg/ml), amphotericin (10 μg), and insulin (10 ng). After 6–7 h, the medium was replaced with fresh serum- and hormone-free medium (1.5 ml/dish), and the cell culture medium was replaced by fresh serum- and hormone-free medium (1 3 ml/dish) on day 2. On day 1, the cells were treated with PAO or the vehicle (0.1% DMSO) for 1 day. Then, the cells were used for the experiments.

**Transfection and Luciferase Assay**—Hepatocytes were cultured at 4 × 10^4 cells per dish (35 × 10 mm) in Williams’ medium E supplemented with 10% serum, dexamethasone (10 nM), and insulin (10 nM) for 1 day. Then, cultured cells were replaced by the medium without serum and hormones and were subjected to transfection with 16 μg of plasmid DNA per 10 cm Petri dish using LipofectAMINE (Life Technologies) and 2 μg of pCMV-LacZ as an internal control and 0.25 μg of pRNOS-Luc; and 0.25 μg of CMV enhancer/promoter-driven β-galactosidase expression plasmid pCMV-LacZ as an internal control and 0.25 μg of plasmid expressing NF-κB (0.125 μg of each pCMV-p65 and pCMV-p50). After a 5-h incubation at 37°C, the medium was replaced by fresh medium supplemented with 10% serum, dexamethasone, and insulin. On day 2, the cells were treated with PAO or the vehicle (0.1% dimethylsulfoxide) for 15 min, washed with the medium, and incubated for 8 h. Activities of luciferase and β-galactosidase were measured as reported previously (20). The luciferase activity was normalized by dividing relative light units by β-galactosidase activity. Transfection was performed in triplicate.

**Statistical Analysis of Data**—Statistical significance was analyzed by Bonferroni/Dunn’s test, and a p < 0.05 was considered to be statistically significant.

**RESULTS**

**Inhibition of Nitric Oxide Production by Arsine Oxide Derivative in Hepatocytes**—As reported previously, an inflammatory cytokine IL-1β increased levels of nitrite (nitric oxide metabolite) released to the medium in primary cultures of rat hepatocytes, where concentrations for the maximal and half-maximal effects were 1 nM and 30 pM, respectively (8). Simultaneous addition of PAO, a trivalent arsenical compound, inhibited the nitrite release stimulated by IL-1β in the time- and concentration-dependent manner (Fig. 1A). Concentration for the maximal inhibition was 2 μM (Fig. 1B). We also observed the similar effect when PAO was pretreated for 15 min and was removed from the medium before experiments instead of simultaneous addition (data not shown). Thus the latter condition was used.

**Construction of Rat iNOS-Luciferase Reporter Gene and Effector Plasmids**—The 5-flanking region of rat iNOS gene (−1026 to +16) were amplified from rat genomic DNA by Polymerase chain reaction (PCR) using primers based on the published sequence (17), pGEM-1CAT CAGGCTGATCGTGAATTGGGAACTATG (KpnI site) and gcaagctc tgaAGATCTCGCTTCAACTCCCTGCT (HindIII site). The product was cloned into pG-B vector harboring promoterless firefly luciferase gene (Boo T-Net Co. Ltd., Japan) to create the 1042-bp rat iNOS promoter-luciferase construct (pRNOS-Luc). pGCGp56 and pCGp105NcoI encoding pRNOS-Luc; and 0.25 μg of CMV enhancer/promoter-driven β-galactosidase expression plasmid pCMV-LacZ as an internal control and 0.25 μg of plasmid expressing NF-κB (0.125 μg of each pCMV-p65 and pCMV-p50). After a 5-h incubation at 37°C, the medium was replaced by fresh medium supplemented with 10% serum, dexamethasone, and insulin. On day 2, the cells were treated with PAO or the vehicle (0.1% dimethylsulfoxide) for 15 min, washed with the medium, and incubated for 8 h. Activities of luciferase and β-galactosidase were measured as reported previously (20). The luciferase activity was normalized by dividing relative light units by β-galactosidase activity. Transfection was performed in triplicate.
in the following experiments. Both trypan blue exclusion and lactate dehydrogenase release tests implied that drug toxicity was not a cause of the decreased production of nitric oxide in response to IL-1β.

Inhibition of the Induction of iNOS Protein and Its mRNA by PAO—IL-1β induced the formation of iNOS protein, which has a calculated molecular mass of 130 kDa (37), with a maximum at 8–12 h as reported (22, 34). PAO inhibited the induction of iNOS protein (Fig. 2). Furthermore, PAO inhibited the induction of iNOS mRNA, which appeared at 3 h after addition of IL-1β and increased to a maximum at 6–8 h (Fig. 3). The results indicated that PAO inhibited IL-1β-stimulated nitric oxide production at a transcription step.

Blockade of PAO-induced Inhibitory Effects on IL-1β-induced iNOS Induction by Vincinal Dithiol Compound—PAO is known to react with two thiol groups of closely spaced protein cysteiny1 residues to form stable dithioarsine rings (29). Interaction between PAO and vincinal dithiol-containing proteins cannot be competed by monothiols, but in the presence of low molecular-weight dithiols such as 2,3-dimercaptopropanol and 1,4-dithiothreitol (DTT) the binding is competed. PAO (2 μM) was treated in the presence of dithiol, 2,3-dimercaptopropanol (50 μM), or monothiol, 3-mercapto-1,2-propanediol (thioglycerol, 50 μM). The 2,3-dimercaptopropanol, but not thioglycerol, abolished PAO-induced inhibitions on the production of nitric oxide and inductions of iNOS protein and its mRNA (Fig. 4). In the case of monothiols, even larger excess of thioglycerol (250 μM) had no effect at all (data not shown).

Effects of PAO on IkB Degradation and NF-κB Activation—IL-1β stimulated a rapid degradation of inhibitory subunits, IkBa and IkBβ proteins, of NF-κB, which were recovered within 1.5 and 4 h, respectively, after addition of IL-1β. PAO had no effect on both IkBa degradation (data not shown). However, EMSA revealed that PAO markedly decreased NF-κB band in nuclear extract prepared without DTT (Fig. 5) although PAO had no effect on IL-1β-induced NF-κB translocation in nuclear extract prepared with DTT. In EMSA experiments for DNA binding activity of NF-κB, we used to prepare nuclear extracts in the presence of DTT. Thus, NF-κB band in the nuclear extract without DTT had lower intensity than that with DTT in control (IL-1β without PAO) (Fig. 5). In the case of nuclear extract with DTT, it could be possible that DTT reversed the binding of PAO to vincinal dithiol-containing residue of NF-κB molecule during the nuclear extraction. These results indicated that PAO could not influence the translocation of NF-κB from the cytoplasm into the nucleus following IkB degradation but could prevent a DNA binding of NF-κB, resulting in the blockade of NF-κB activation induced by IL-1β in hepatocytes.

Evidence for Interaction between PAO and NF-κB—In the next, after NF-κB activation by IL-1β in the absence of PAO, nuclear extracts were prepared without DTT, and then PAO were incubated in nuclear extracts in the absence or presence of DTT or thioglycerol in vitro. Addition of exogenous PAO again decreased NF-κB band in the absence of DTT but not in the presence of DTT (Fig. 6). We also found the similar decrease of NF-κB band in the nuclear extract with monothiol, thiogly-
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absence of dithiothreitol (DTT) in the absence and presence of dithiothreitol (DTT) and were incubated with [γ-32P]ATP-labeled NF-κB consensus oligonucleotide, followed by electrophoresis and analysis by autoradiography.

In the present study, we found that arsine oxide derivative PAO inhibited the induction of nitric oxide and the induction of IL-1B through its vicinal dithiol moiety. In support of this observation, Shumilla et al. (28) reported that arsenite as well as other thiol reactive metals inhibited NF-κB binding to DNA in vitro with nuclear extracts isolated from TNFα-treated A549 cells, through interaction with critical protein sulphydryls, which was reversed by addition of dithiols, DTT. Kim and Stadtman (41) reported the similar mechanism for inhibitions of NF-κB DNA binding and nitric oxide induction by selenite in human Jurkat T cells and lung adenocarcinoma cells. Selenite inhibited NF-κB activation via adduct formation with the essential thiols of this transcription factor. Selenite inhibition was also reversed by addition of DTT. Inhibition because of formation of such selenotrisulfide type of adduct is established as the mechanism of selenite action in the case of rat brain prostaglandin D synthase (42).

It has been reported that PAO is an inhibitor of a specific class of tyrosine phosphatases characterized by two vicinal thiol groups in the active site (43–45). Singh and Aggarwal (46) reported that PAO (2.4 μM) inhibited the degradation of IκB protein and NF-κB activation induced by TNFα in human myeloid ML-1a cells, which were reversed by DTT, suggesting a critical role of sulphydryl group. They concluded that PAO presumably inhibited tyrosine phosphatase, which occurs in the cytoplasm and is involved in the pathway leading to the activation of NF-κB. They had no mention about interaction between PAO and NF-κB. We cannot exclude the possibility that PAO inhibited tyrosine phosphatase in our study, which occurs in the nucleus and is involved in NF-κB DNA binding. However, it seems that different types of cells have alternative pathways for the activation of NF-κB (47). PAO may attack different targets which have a vicinal dithiol moiety and are involved in NF-κB activation. Tewes et al. (48) reported that PAO inhibited IL-1-induced activation of IL-1R1 associated protein kinase and impaired the activation of NF-κB in a murine T cell line, EL4 cells.

In our primary cultured hepatocytes, we found that pretreatment of sodium arsenite (20 μM for 15 min) also markedly inhibited the production of nitric oxide and the induction of...
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iNOS protein and mRNA by IL-1β as similar as PAO did. However, addition of DTT (500 μM) could not reverse the inhibition. Furthermore, sodium arsenite had no effect on iNOS promoter activation with p50/p65 at all,2 implying that PAO and sodium arsenite inhibits iNOS induction via different mechanisms. de Vera et al. (27, 49) reported that sodium arsenite inhibited iNOS expression induced by a mixture of cytokines and lipopolysaccharide in rat and human hepatocytes through the induction of Hsp-72, which possibly attenuated the transcription level via the inhibition of NF-κB. Although a precise role of vicinal dithiol residue in NF-κB activation is obscure on the induction of iNOS in hepatocytes, our study demonstrates that vicinal dithiol residue(s) which interacts with PAO may be an essential component for the binding to iNOS promoter region. Thus, maintenance of active thiol forms with PAO may attack the multi-molecules which are regulated by changes in the redox state.

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