Inhibition of CDKs by roscovitine suppressed LPS-induced •NO production through inhibiting NFκB activation and BH4 biosynthesis in macrophages

Jianhai Du¹,²,³*, Na Wei¹,²,⁴*, Tongju Guan¹,²,³, Hao Xu¹,²,³, Jianzhong An¹,²,³, Kirkwood A Pritchard, Jr.¹,²,³ and Yang Shi¹,²,³

¹Division of Pediatric Surgery, Department of Surgery,
²Children’s Research Institution,
³Cardiovascular Research Center
Medical college of Wisconsin, 8701 Watertown Plank Road,
Milwaukee, WI 53226, USA

⁴Qilu Hospital of Shandong University, Jinan, China

*Contribute equally to this work.

Surname and Short title: Du et al, Roscovitine inhibited LPS-induced •NO production

Correspondence to: Yang Shi, PhD, or Jianhai Du, PhD, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226. E-mail: yangshi@mcw.edu or jhdu@mcw.edu

Total Word count: 6155

Word count of abstract: 250
ABSTRACT

In inflammatory diseases, tissue damage is critically associated with nitric oxide (•NO) and cytokines which are overproduced in response to cellular release of endotoxins. Here we investigated the inhibitory effect of roscovitine, a selective inhibitor of cyclin-dependent kinases (CDKs) on •NO production in mouse macrophages. In RAW264.7 cells we found that roscovitine abolished the production of •NO induced by lipopolysaccharide (LPS). Moreover, roscovitine significantly inhibited LPS-induced inducible nitric oxidase synthase (iNOS) mRNA and protein expression. Our data also showed that roscovitine attenuated LPS-induced phosphorylation of IKKβ, IκB and p65 but enhanced the phosphorylation of ERK, p38 and JNK. In addition, roscovitine dose-dependently inhibited LPS-induced expression of COX-2, IL-1β and IL-6 but not TNFα. Tetrahydrobiopterin (BH4), an essential cofactor for iNOS, is easily oxidized to 7, 8-dihydrobiopterin (BH2). Roscovitine significantly inhibited LPS-induced BH4 biosynthesis and decreased BH4/BH2 ratio. Furthermore, roscovitine greatly reduced the upregulation of GTP cyclohydrolase 1 (GCH-1), the rate-limiting enzyme for BH4 biosynthesis. Using other CDK inhibitors, we found that CDK1, CDK5, and CDK7, but not CDK2, significantly inhibited LPS-induced •NO production in macrophages. Similarly, in isolated peritoneal macrophages, roscovitine strongly inhibited •NO production, iNOS and COX-2 upregulation, activation of NFκB and induction of GCH-1 by LPS. Together, our data indicates that roscovitine abolishes LPS-induced •NO production in macrophages by suppressing NFκB activation and BH4 biosynthesis, which might be mediated by CDK1, CDK5 and
CDK7. Our results also suggest that roscovitine may inhibit inflammation and that CDKs may play important roles in the mechanisms by which roscovitine attenuates inflammation.

**Keyword:** roscovitine; nitric oxide (•NO); tetrahydrobiopterin (BH4); NFκB; macrophage
INTRODUCTION

Macrophages actively participate in host defense and inflammation. When exposed to Gram-negative bacteria, macrophages are activated by lipopolysaccharide (LPS) in the bacterial cell wall, which triggers the release of large amounts of nitric oxide (•NO) and inflammatory cytokines. The overproduced •NO may destroy the bacteria but may also cause tissue injury and toxicity in the host by its derivatives (22). •NO is a free radical produced from L-arginine by at least three •NO synthases (NOS). iNOS is the inducible isoform also known as NOS2, which is responsible for the production of high levels of •NO in macrophages induced by LPS. By interacting with Toll-like receptor 4 (TLR4), LPS activates intracellular signaling pathways including the Mitogen-activated protein kinase (MAPK) and nuclear factor κB (NFκB) pathway to upregulate iNOS expression in macrophages (7, 8, 19, 20). The NFκB pathway is the most important signaling pathway in TLR4-mediated iNOS induction and inflammatory response. The p50 (NFκB1)/p65 (RelA) heterodimer is the most abundant form of NFκB. Phosphorylation of NFκB p65 (serine 536) is physiologically induced in response to a variety of proinflammatory stimuli. Phosphorylation of p65 is critical in regulating the activation, nuclear localization and transcription activity of NFκB.

Tetrahydrobiopterin (BH4) is an essential cofactor for all NOS isoforms and has been shown to be an important regulator of iNOS activity and iNOS dependant •NO production (1, 4, 28). GTP cyclohydrolase I (GCH1) is the first step and the rate-limiting enzyme for BH4 biosynthesis in the de novo pathway. GCH1
transgenic mice showed significantly enhanced renal iNOS expression and •NO levels with LPS treatment (36). Inhibition of GCH1 suppressed LPS-induced •NO production (15) and IFNγ-induced iNOS expression (5).

In our recent study on regulation of GCH1 by phosphorylation using site-directed mutagenesis, we found that the mutation of threonine at 85 (T85) of rat GCH-1 into alanine (mimic dephosphorylation and loss of function) could completely inhibit GCH1 activity and BH4 production (unpublished). Since T85 on GCH1 has the consensus sequence for cyclin-dependent kinase 5 (CDK5), we originally hypothesized that inhibition of CDK5 may suppress GCH1 phosphorylation and, accordingly, •NO production in macrophages. However, our results indicated that CDK5 inhibitor roscovitine directly inhibited GCH1 expression (mRNA and protein) induced by LPS. Therefore, we hypothesize that roscovitine inhibits LPS-induced •NO production by inhibiting BH4 synthesis and suppressing LPS-activated NFκB pathway and/or MAPK pathway. Accordingly, the aim of this study is to explore the inhibitory effect of roscovitine on the expression of iNOS and GCH1, production of •NO and BH4, and activation of NFκB pathway and MAPK pathway in macrophage by LPS stimulation.

MATERIALS AND METHODS

Materials. LPS, roscovitine, CDK7 inhibitor (5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside) and anti-actin antibody were purchased from Sigma (St. Louis, MO). CDK1 inhibitor (3-(2-Chloro-3-indolylmethylene)-1,3-dihydroindol-2-one),
CDK2/5 inhibitor (N^4-(6- Aminopyrimidin-4-yl)-sulfanilamide) and CDK2 inhibitor II (compound 3) were purchased from Calbiochem (San Diego, CA). Anti-GCH1 antibody was generously provided by Dr. Zvonimir S. Katusic (29). Antibodies against phospho-ERK, phospho-p38, phospho-JNK, phospho-P65, P65, phospho-IκB, and phospho-IKK were from Cell Signaling Technology (Boston, MA). Antibodies against iNOS and GAPDH were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-COX-2 was from Cayman Chemical (Ann Arbor, MI).

**Cell culture.** Mouse macrophage cell line RAW264.7 cells were purchased from American Type Tissue Culture Collection (ATCC, Rockville, MD). RAW264.7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were plated in 12-well plates at a density of 1×10^6 cells/well for •NO assay and BH4 assay, and plated in 60 mm-plates at a density of 5×10^6 cells/well for other studies. All culturing was performed in a humidified atmosphere under 5% CO₂ at 37°C.

**Peritoneal macrophage isolation and culture.** The discarded bodies of Dahl S rats from another study were used. After the rat heart was removed for other studies, we promptly opened the peritoneal cavity and washed it with 10 ml pre-warmed 1640 medium twice. The eluted macrophages were centrifuged for 10 minutes at 300×g. The macrophages were resuspended with 1640 medium containing 20% FBS and seeded onto 60-mm plates at 8×10^6 cells each plate. After two hours of plating, the unattached cells were washed off by PBS and the peritoneal macrophages were incubated with 1640 medium containing 10% FBS for all experiment.
RNA extraction and Realtime (RT)-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen) and treated with DNase I using DNA-Free kit (Ambion). cDNA was generated from 1 µg of total RNA by using iScript™ cDNA Synthesis Kit (Bio-Rad). The primers for RT-PCR were synthesized by Operon Biotechnologies, Inc (Huntsville, AL). The primer sequences for iNOS were 5'-CTG CTT TGT GCG AAG TGT CAG T-3' (forward) and 5'-GGC ACC CAA ACA CCA AGC TC-3' (reverse); the primer sequences for GCH-1 were: 5'–GGC CGC TTA CTC GTC CAT CCT-3' (forward) and 5'-GGT CTC CTG GTA TCC CTT GGT GAA-3' (reverse); the sequences for IL-1β were 5'-GTG GCA GCT ACC TGT GTC TT-3' (forward) and 5'-GGA GCC TGT AGT GCA GTT GT-3' (reverse); the sequences for IL-6 were 5'-GGA GCC TGT AGT GCA GTT GT-3' (reverse); and the sequences for TNFα were 5'-GGA GCC TGT AGT GCA GTT GT-3' (reverse). RT-PCR was performed on iCycler iQ Real Time PCR instrument (Bio-Rad) by duplicate using iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instruction. The reaction for GAPDH mRNA was amplified for all samples as the internal reference. The amplification conditions were initial denaturation at 95°C followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C at 30 s and elongation at 72°C for 1 min. If the difference in the cycle (Ct) values between the target gene (GCT-1 or iNOS, etc.) and the reference gene (GAPDH) is defined as ΔCt (Ct$_{\text{target gene}}$–Ct$_{\text{GADPH}}$), then mRNA expressions were
calculated as $2^{\Delta Ct}$ and expressed by fold increase over the unstimulated macrophages ($2^{\Delta Ct_{LPS}}/2^{\Delta Ct_{no-LPS}}$).

• **NO measurement.** The Nitrite ($NO_2^-$) was determined by the Griess Reagent System (Promega, Madison WI). Briefly, 50 µl of medium from cells with or without treatment was dispensed into 96-well plate. After incubation with sulfanilamide solution (50 µl) for 10 min at R.T., the medium was then mixed with NED solution (50 µl) for 10 min at R.T. The color development was assessed at $\lambda = 550$ nm with a microplate reader. Fresh culture medium was used as the blank and the amount of nitrite in the samples was calculated from a sodium nitrite standard curve using freshly prepared nitrite standards (0–100 µM).

**BH4 and BH2 assay.** BH4 and BH2 were assayed by using HPLC with an electrochemical detector (ESA Biosciences CoulArray® system Model 542). Cell pellets were immediately lysed by passing 28-gauge tuberculin syringe in 150 µl 50 mM phosphate buffer pH 2.6 containing 0.2 mM DTPA and 1 mM DTE (freshly added). Samples were centrifuged at 12000 x $g$ for 10 min at 4°C, and supernatants were analyzed using a Synergi Polar-RP column eluted with argon saturated 50 mM phosphate buffer (pH 2.6). Multi-channel coulometric detection was set between 0-600 mV. One channel was set at -250 mV to verify the reversibility of BH4 oxidative peak detection. Calibration curves were made by summation of the peak areas collected at 0 and 150 mV for BH4 and 280 and 365 mV for BH2. Intracellular concentrations were calculated using authentic BH4 and BH2 standards. BH4 and BH2 were then normalized by the cells’ protein concentrations (12).
Western blot analysis. Western blot analysis was performed as we previously reported (33). Briefly, 30 µg of cell lysates was loaded into and separated by 12% SDS/PAGE and incubated with primary antibodies overnight and secondary antibody for 1 hour. The blotted membranes were visualized with Super Signal West Pico kit (Pierce Biotechnology.)

Statistics. Data are expressed as mean ± SEM and were analyzed by two-way ANOVA or by two-tailed t-test. A value of $P< 0.05$ was considered statistically significant.

RESULTS

Roscovitine inhibited the •NO production, iNOS expression and reactive oxygen species (ROS) generation induced by LPS. To determine the potential cell cytotoxicity of roscovitine, RAW 264.7 macrophages were treated with roscovitine at 1 µM, 10 µM and 25 µM for 24 hours, and cell viability was measured by MTT assay. As shown in Supplement Figure I, roscovitine at all concentrations didn’t significantly affect cell viability, although roscovitine at 25 µM slightly decreased viable cells. These data indicate that roscovitine has little cytotoxicity at the concentrations we were using on macrophages. Interestingly, roscovitine at 25 µM considerably reduced the viable cells in LPS-treated cells, indicating that roscovitine may induce apoptosis in inflammatory macrophages (Supplement Figure IB). To determine the effects of roscovitine on •NO production in macrophages challenged with LPS, macrophages were pretreated with roscovitine at the above concentrations (1 µM, 10 µM and 25 µM) for 30 min followed by stimulation with LPS (2 µg/ml) for 12 hours. As expected, LPS
Du et al, Roscovitine inhibited LPS-induced •NO production

significantly increased the nitrite production which was increased from 1.3±0.7 to 37.4±4.1 µM in the culture medium. Roscovitine at 1 µM did not affect cells •NO production but 10 µM and 25 µM of roscovitine dose-dependently inhibited •NO production from macrophages while roscovitine at 25 µM alone had no effect (Figure 1A). To determine whether roscovitine would also inhibit •NO production by LPS at concentration comparable to physiological conditions, we treated the cells with LPS (100 ng/ml) for 12 hours and determined •NO production. LPS at 100 ng/ml significantly increased •NO production (Supplement Figure III). Similarly, roscovitine at 25 µM reversed the induction back to basal levels. Since iNOS is the major NOS isoform responsible for the •NO production by LPS in macrophages, we further examined the mRNA and protein expression of iNOS. Similarly, roscovitine at 10 µM and 25 µM dose-dependently inhibited the upregulation of iNOS mRNA and protein induced by LPS; however roscovitine at 25 µM did not completely reduce the iNOS upregulation back to the baseline. Excessive generation of ROS is another marker of LPS-induced proinflammatory response. Intriguingly, roscovitine also significantly inhibited LPS-induced ROS production in macrophages (Supplement Figure IV).

**Roscovitine inhibited LPS-induced NFκB activation.** As shown in Figure 2, phosphorylation of p65 was detected and maximized at 15 min after LPS treatment and lasted throughout 180 min. Roscovitine had no obvious effect on LPS-induced p65 phosphorylation at 15 min, but did significantly inhibit LPS-induced p65 phosphorylation from 30 min to 180 min. However, the total p65 expression was not affected by roscovitine. The phosphorylation of IκB may result in its ubiquitinization...
which in turn activates the NFκB. Indeed, we found that IκB was phosphorylated by LPS with a similar pattern as p65, and roscovitine could strongly inhibit the phosphorylation at 30, 60 and 180 min (Figure 2). To confirm the inhibitory effect of roscovitine on the NFκB pathway, we also checked IKK, a kinase upstream of IκB, which regulates the phosphorylation of IκB. We found IKKβ rather than IKKα was phosphorylated by LPS at 15 min and the phosphorylation gradually attenuated. Roscovitine had no effects on IKKβ phosphorylation at 15 min but inhibited IKKβ phosphorylation at 30, 60 and especially 180 min, showing a similar inhibitory pattern as IκB and p65 phosphorylation. It is noteworthy that the phosphorylation of IκB by LPS seems to be biphasic between 15 and 60 min (Figure 2), however no similar patterns of phosphorylation of P65 and IKKβ were observed at the same time period.

Roscovitine inhibited COX-2, IL-1β and IL-6 but not TNFα expression induced by LPS. To further determine the role of roscovitine on NFκB inhibition, we examined the expression of certain genes in response to NFκB activation including COX-2, IL-1β, IL-6 and TNFα. We found that roscovitine dose-dependently inhibited COX-2 protein expression at 10 µM and 25 µM. Roscovitine at 25 µM alone did not affect the COX-2 expression (Figure 3A). RT PCR results showed that LPS significantly increased IL-1β and IL-6 mRNA expressions. Roscovitine at 10 µM and 25 µM, but not at 1 µM, dramatically inhibited the upregulation of IL-1β and IL-6 in a dose-dependent manner. However, roscovitine was not able to inhibit TNFα induction by LPS (Figure 3D). On the contrary, roscovitine alone or with LPS further increased TNFα mRNA expression.
Roscovitine enhanced the activation of MAPK induced by LPS. To determine whether roscovitine could inhibit MAPK, cells were treated with roscovitine (25 µM, 30 min) and then stimulated with LPS (2 µg/ml) for 5, 15, 30, 60 and 180 min. Upon LPS stimulation, phosphorylation of ERK (P44 and P42 MAPK) became evident at 5 min and maximized at 30 min, which then tapered off towards 180 min. However, roscovitine did not inhibit the activation of ERK induced by LPS. In contrast, it further increased and sustained the LPS-induced phosphorylation of ERK at 30 min and 60 min (Figure 4). In the same trend, roscovitine enhanced the LPS-induced p38 phosphorylation at 15 min and 30 min as well as LPS-induced JNK phosphorylation within 0-180 min. These data suggest that roscovitine did not inhibit, but potentiated, the LPS-induced MAPK activation, indicating that MAPK may not be involved in the inhibitory effect of roscovitine on iNOS and •NO induced by LPS.

Roscovitine inhibited BH4 production and GCH-1 expression. After 12 hours treatment, LPS increased BH4 levels in the cells more than 6 fold. Roscovitine (25 µM) strongly inhibited the LPS-induced BH4 to a level even below the control levels; however roscovitine alone had no effect on BH4 production (Figure 5). Interestingly, no significant difference was observed for BH2 levels with roscovitine treatment although roscovitine alone had a tendency to increase BH2 levels. Therefore, roscovitine also significantly reduced the BH4/BH2 ratio, which was indicated to be just as important for NOS coupled activity as total BH4 concentration (9, 34). LPS also noticeably increased the GCH-1 mRNA and protein expression. Pretreatment with roscovitine (25 µM) significantly suppressed the induction of GCH-1 expression
Du et al, Roscovitine inhibited LPS-induced •NO production by LPS (Figure 6).

Inhibition of CDK1, CDK5 and CDK7 but not CDK2 partially inhibited LPS-induced •NO production. As roscovitine is reported to inhibit CDK1, CDK5 and CDK7, we used different CDK inhibitors with different concentrations based on their IC$_{50}$ (Figure 7). CDK1 inhibitor did not inhibit •NO production at 10 μM but dose-dependently inhibited •NO production between 25-50 μM. CDK2 inhibitor did not inhibit •NO production; however, inhibition of both CDK2 and CDK5 strongly suppressed LPS-induced •NO production, indicating that CDK5 but not CDK2 mediates this inhibitory effect. In addition, CDK7 inhibitor inhibited LPS-induced •NO production at 10 and 50 μM at the same degree. All the inhibitors showed a slightly inhibitory effect on cell proliferation at high concentrations except for CDK7 inhibitor which did not affect cell viability at all concentrations (Supplement Figure II).

Inhibition of inflammatory response, NFκB activation and GCH-1 expression in isolated peritoneal macrophages. RAW264.7 macrophage cell line was established from a tumor induced by Abelson murine leukemia virus. How this might affect CDKs signaling remains unclear. To overcome the limitation that data from using RAW264.7 macrophage cells may not be able to fully represent the macrophages without modification, rat peritoneal macrophages were isolated and used for examining the effects of roscovitine on LPS-induced inflammatory response. Roscovitine (25 μM) significantly inhibited •NO production and induction of iNOS and COX-2 stimulated by LPS (1 μg/ml, 12 hours) in peritoneal macrophages (Figure 8A and 8B). Moreover, roscovitine also obviously decreased the upregulation of GCH-1 by LPS, indicating
that BH4 synthesis is also inhibited by roscovitine (Figure 8C). As phosphorylation of p65 and IκB in RAW264.7 cells were evident at 30 min, we stimulated peritoneal macrophages with LPS (1 µg/ml) for 30 min and demonstrated that the phosphorylation of P65 and IκB was markedly elevated which was decreased by roscovitine pretreatment (25 µM, 30 min) (Figure 8D).

**DISCUSSION**

The major findings of this study are that roscovitine inhibited LPS-induced iNOS production of •NO through attenuating the activation of the IKK/IκB/NFκB pathway and suppressing GCH1-dependent BH4 production. We also found that inhibition of CDK1, CDK5 and CDK7, but not CDK2, significantly decreased LPS-induced •NO production, indicating that CDK1, CDK5 and CDK7 mediate macrophage activation by LPS.

Roscovitine is a purine analogue that selectively inhibits CDKs by binding to their active sites. Currently, roscovitine is being studied under phase II human trials as an anticancer agent (32). Recently it was shown that roscovitine also possesses anti-inflammatory properties. Roscovitine appears to resolve inflammation by promoting apoptosis in inflammatory cells, a finding that was confirmed in murine models of lung injury and arthritis (30). Roscovitine may also down-regulate NFκB activation in response to TNFα and interleukin 1 in A549 and 293 cells (11). During preparation of this manuscript, a study similar to ours showed that roscovitine down-regulated NFκB and inhibited iNOS expression by LPS in macrophages (18). In agreement with this report, our results showed that roscovitine attenuated NFκB
activation as shown by decreased phosphorylation of p65, IκB and IKKβ in both RAW264.7 macrophages and primary macrophages (Figure 4 and Figure 8). Furthermore, roscovitine was found to be able to abolish the generation of ROS acutely induced by LPS (Supplement Figure IV). LPS activates NFκB by the canonical pathway: phosphorylation of IκB by IKK triggers the degradation of IκBα to activate NFκB p65 subunit (23). As we did not detect any increase of IKKα phosphorylation by LPS, we think it is possible that IKKβ is the major player in the activation of IκB phosphorylation. Activation of IKKβ is essential for the canonical pathway (16). Recently, roscovitine was shown to inhibit IKK/NFκB in sensitizing TRAIL-induced apoptosis in thyroid carcinoma cells (13). Interestingly, we observed a transient drop in IκB phosphorylation induced by LPS at 30 min which was not observed in either IKKβ or p65 activation. Moreover, roscovitine showed significant inhibition of LPS-induced NFκB phosphorylation after 15 min. This suggests that LPS-induced NFκB activation has two phases, with the later being regulated by CDKs.

After dissociating from IκB, NFκB translocates to the nucleus to induce expression of downstream target genes (23). It is well accepted that LPS up-regulates the expression of COX-2, IL-6, IL-1β and TNFα, in part because of the presence of an NFκB binding site in their promoters. Interestingly, roscovitine dose-dependently inhibited LPS-induced COX-2, IL-6 and IL-1β expression yet increased TNFα expression (Figure 6). As inhibition of ERK and JNK also block TNFα production (10, 25), we speculate that increased TNFα expression is due to
Du et al, Roscovitine inhibited LPS-induced •NO production

roscovitine activating MAPK (Figure 4), which has been shown in keratinocytes (2), and human colon cancer cell lines (37, 38). The increased TNFα may also contribute to the pro-apoptotic effect of roscovitine on inflammatory cells (Supplement Figure IB).

The iNOS promoter contains both NFκB and AP-1 binding sites. Roscovitine significantly inhibited iNOS and •NO, indicating that roscovitine repressed iNOS by inhibiting the NFκB pathway but not the MAPK pathway which was instead activated. It will be intriguing to speculate that crosstalk between these pathways work together to modulate •NO production.

iNOS activity is also positively regulated by BH4, which stabilizes dimer formation (28). In macrophages, a deficiency in BH4 impaired •NO production after LPS stimulation (31). In our study, roscovitine significantly inhibited LPS-induced BH4 production and decreased the BH4/BH2 ratios. At 25 µM of roscovitine, although iNOS expression was inhibited it was still ~4 fold higher than the basal levels. Yet, LPS-induced •NO production was reduced to unstimulated levels. This demonstrates just how important BH4 is for optimal iNOS activity. As the major rate-limiting enzyme in BH4 biosynthesis, GCH1 could be regulated in cells and tissues at the level of transcription and post-translational modification (39). Inhibition of phosphoinositide 3-kinase (PI3K) counteracted LPS-induced GCH1 expression in macrophages (27). In PI3K-deficient macrophages, the induction of GCH1 and biosynthesis of BH4 were greatly reduced (31). Interestingly, PI3K was recently found to be upstream of CDK5 and CDK5 can be induced by brain-derived neurotrophic
factor (6) and insulin (21). In contrast, when CDK5 activity is inhibited, it also attenuates PI3K activation in neuroblastoma cells (35). It remains to be determined whether CDK5 is upstream or downstream of PI3K in macrophages. Recently cytokine induction of GCH1 was shown to require coordinated activation of NFκB and Stat1/Stat3 in endothelial cells to increase BH4 production (17). In other studies it was discovered that CDK5 phosphorylates STAT3 at Ser 727 \textit{in vitro} and \textit{in vivo} to increase STAT3 activation (14).

Among 151 tested protein kinases, roscovitine did not affect most of the protein kinases but was observed to be a selective inhibitor of CDKs (3). Roscovitine was also reported to inhibit CDK1, CDK2, CDK7 and CDK9 and could bind CDK2 (26) and CDK5 (24). Our data show that LPS-induced •NO production was partially inhibited by CDK1, CDK7 and CDK2/5 inhibitors, but not the CDK2 inhibitor, indicating that CDK1, CDK5 and CDK7 (but not CDK2) are involved in the mechanism of inhibitory action of roscovitine.

In conclusion, we have shown that roscovitine abolishes LPS-induced •NO production in macrophages by suppressing BH4 biosynthesis and NFκB activation. The inhibitory effect of roscovitine appears to be through its ability to inhibit CDK1, CDK5 and CDK7. Our study suggests that roscovitine may inhibit inflammation and that CDKs may play important roles in the mechanisms by which roscovitine attenuates inflammation.
ACKNOWLEDGEMENTS

The administrative assistance of Meghann Sytsma (BS) and Anne Laulederkind (BSN, BA) are gratefully acknowledged.

GRANTS

This study was supported by grant HL080468 (to Y. S.) from the National Institutes of Health, Bethesda, Maryland.
FIGURE LEGENDS

Figure 1. Roscovitine inhibited iNOS expression and •NO production. Cells were pretreated with roscovitine at different concentrations for 30 min before stimulated with LPS (2 µg/ml) for 12 hours. Supernatant was collected for (A) nitrite production and cells were lysed for (B) real-time PCR of iNOS mRNA expression or (C) western blot analysis of iNOS protein expression. Results were from 4 separate experiments. #P<0.01 vs. Control and *P<0.01 vs. LPS alone.

Figure 2. Roscovitine inhibited the NFκB activation by LPS. Cells were pretreated with roscovitine (25 µM) for 30 min before incubated with LPS at different times as shown above. The immunoblot of cell lysate was probed with antibodies against phospho-IκB, phospho-p65 and phospho-IKK, and reprobed with antibodies against p65 or actin. The results are representative of four blots.

Figure 3. Roscovitine inhibited COX-2, IL-1β and IL-6 induction by LPS. Cells were pretreated with roscovitine for 30 min followed by LPS stimulation for 12 h. (A) The cell proteins were immunoblotted with antibodies against COX-2 and actin, and the RNA was extracted to determine (B) IL-1β and (C) IL-6 mRNA expression by real-time PCR. Results were from 4 separate experiments. #P<0.01 vs. Control and *P<0.01 vs. LPS alone.

Figure 4. Roscovitine did not inhibit MAPK activation by LPS. Cells were pretreated with roscovitine (25 µM) for 30 min before incubated with LPS at different times as shown. The immunoblot of cell lysate was probed with antibodies against phospho-ERK, phospho-JNK and phospho-p38, and reprobed with antibodies against
GAPDH. The results are representative of four blots.

**Figure 5.** Roscovitine inhibited BH4 production by LPS. Cells were pretreated with roscovitine (25 µM) for 30 min before incubated with LPS for 12 hours. Cells were lysed in acidic buffer (see materials and methods) and analyzed by HPLC for BH4 (Figure 5A) and BH2 (Figure 5B) levels. The ratio of BH4/BH2 was shown in Figure 5C. The experiments were repeated 3 times. #P<0.01 vs. Control and *P<0.01 vs. LPS alone.

**Figure 6.** Roscovitine inhibited GCH-1 expression. Cells were pretreated with roscovitine for 30 min before stimulated with LPS for 12 hours. Cells were lysed for (A) real-time PCR of GCH-1 mRNA expression or (B) immunoblot of GCH-1 protein expression by probing against antibodies against GCH-1 and actin. Results were from 4 separate experiments. #P<0.01 vs. Control and *P<0.01 vs. LPS alone.

**Figure 7.** The effects of CDK inhibitors on •NO production. Cells were pretreated with different CDK inhibitors at different concentrations as indicated for 30 min before stimulated with LPS for 12 hours. Supernatant was collected for nitrite production. Results were from 3 separate experiments. *P<0.01 vs. LPS alone.

**Figure 8.** The effects of roscovitine on inflammatory response, NFκB activation and GCH-1 expression in primary macrophages. Rat peritoneal macrophages were pretreated with roscovitine (25 µM, 30 min) and stimulated with LPS (1 µg/ml) for 12 hours. The supernatant was collected to assay nitrite concentrations (A) and the cells were lysed to examine the protein expressions of GCH-1 (B), iNOS and COX-2 (C) by western blot analysis. (D)The macrophages were stimulated with LPS for 30 min to
determine the phosphorylation of P65 and IκB by western blot analysis.

SUPPLEMENT

Materials and Methods

Cell viability assay

Cells were seeded at 1×10^4 cells/well in 96-well plates. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL; Sigma, St Louis, MO, USA) was added to each well and incubated for 4 h at 37°C. The formazan product was dissolved by adding 150 µL DMSO to each well. The MTT absorbance value was detected at 490 nm by the microplate reader.

ROS Detection

ROS production was detected by using Lucigenin-enhanced Chemiluminescence. Macrophages were seeded in 35-mm culture dish. With/without pretreatment with roscovitine (25 µM) for 30 min, the cells were stimulated with or without LPS (2 µg) for 5 min. After addition of lucigenin (5 µM) into the dish, the cells were placed in a luminometer (Turner biosystems) to measure chemiluminescence for 5 min. The dish containing only the medium and lucigenin was read as background luminescence, and this value was subtracted from each sample. The generation of ROS was presented as relative light units (RLU).

Supplement Figure legends
Supplement Figure I. The effect of roscovitine on cell viability. (A) Cells were treated with roscovitine at different concentrations for 24 hours. (B) Cells were treated with roscovitine (25 µM), LPS (2 µg/ml) or both for 12 hours. Cell viability was determined by MTT. Data are presented as mean±SEM of triple determinations. The experiment was repeated three times.

Supplement Figure II. The effects of CDK inhibitors on cell viability. Cells were incubated with CDK inhibitors at different concentrations for 24 hours to determine cell viability by MTT. *P<0.01 vs. control. Data are presented as mean±SEM of triple determinations. The experiment was repeated three times.

Supplement Figure III. The effect of roscovitine on •NO production by LPS at concentration comparable to physiological conditions. Cells were pretreated with roscovitine (25 µM) for 30 min before stimulated with LPS (100 ng/ml) for 12 hours. Supernatant was collected for nitrite production. #P<0.01 vs. Control and *P<0.01 vs. LPS alone (N=4).

Supplement Figure IV. The effect of roscovitine on ROS production by LPS. Cells were pretreated with roscovitine (25 µM) for 30 min and then incubated with LPS (2 µg/ml) for 5 min. The chemiluminescence was measured by a luminometer. #P<0.01 vs. Control and *P<0.01 vs. LPS alone (N=3).
REFERENCES


27. Ota A, Kaneko YS, Mori K, Nakashima A, Nagatsu I, and Nagatsu T. Effect of peripherally administered lipopolysaccharide (LPS) on GTP cyclohydrolase I,


Figure 1

A B

C

Figure 1
Figure 2
Figure 3

(A) Western blot analysis showing COX-2 and Actin expression under Roscovitine (μM) and LPS treatment.

(B) Bar graph showing IL-1β mRNA expression (Fold) under Roscovitine (μM) and LPS treatment.

(C) Bar graph showing IL-6 mRNA expression (×1000 Fold) under Roscovitine (μM) and LPS treatment.

(D) Bar graph showing TNFα mRNA expression (Fold) under Roscovitine (μM) and LPS treatment.
Figure 4
Figure 5

A. Bar graph showing BH4 (pmol/mg protein) levels with different conditions.

B. Bar graph showing BH2 (pmol/mg protein) levels with different conditions.

C. Bar graph showing BH4/BH2 (Ratio) levels with different conditions.

Legend:
- Roscovitine
- LPS
- -
- +

Significance:
- *
- #
**Figure 6**

(A) Bar graph showing GCH-1 mRNA (Fold) levels in response to varying concentrations of Roscovitine and LPS. The data points are indicated with error bars. Significant changes are marked with asterisks (*).

(B) Western blot analysis of GCH-1 expression under different conditions. The blot shows a band at the expected molecular weight of GCH-1, with Actin as a loading control.
Figure 7

A

B

CDK1 inhibitor (μM) - - 10 25 50
LPS

CDK2 inhibitor (μM) - - 0.1 1 10
LPS

CDK2/5 inhibitor (μM) - - 5 10 50
LPS

CDK7 inhibitor (μM) - - 1 10 50
LPS

Figure 7
Figure 8
Supplement Figure II

A) Cell viability (OD value) against CDK1 inhibitor (µM) concentrations: 0, 10, 25, 50.

B) Cell viability (OD value) against CDK2 inhibitor (µM) concentrations: 0, 0.1, 1, 10.

C) Cell viability (OD value) against CDK2/5 inhibitor (µM) concentrations: 0, 10, 50, 100.

D) Cell viability (OD value) against CDK7 inhibitor (µM) concentrations: 0, 1, 10, 20.
Supplement Figure III