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Inhibition of CDKS by roscovitine suppressed LPS-induced •NO production through inhibiting NFκB activation and BH_4 biosynthesis in macrophages

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Du J, Wei N, Guan T, Xu H, An J, Pritchard KA, Jr., Shi Y. Inhibition of CDKS by roscovitine suppressed LPS-induced •NO production through inhibiting NFκB activation and BH_4 biosynthesis in macrophages. *Am J Physiol Cell Physiol* 297: C742–C749, 2009. First published June 24, 2009; doi:10.1152/ajpcell.00138.2009.—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 oxide synthase (iNOS) mRNA and protein expression. Our data also showed that roscovitine attenuated LPS-induced phosphorylation of IκB kinase β (IKKβ), IκB, and p65 but enhanced the phosphorylation of ERK, p38, and c-Jun NH_2-terminal kinase (JNK). In addition, roscovitine dose dependently inhibited LPS-induced expression of cyclooxygenase-2 (COX)-2, IL-1β, and IL-6 but not tumor necrosis factor (TNF)-α. Tetrahydrobiopterin (BH_4), an essential cofactor for iNOS, is easily oxidized to 7,8-dihydrobiopterin (BH_2). Roscovitine significantly inhibited LPS-induced BH_4 biosynthesis and decreased BH_4-to-BH_2 ratio. Furthermore, roscovitine greatly reduced the upregulation of GTP cyclohydrolase-1 (GCH-1), the rate-limiting enzyme for BH_4 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 indicate that roscovitine abolishes LPS-induced •NO production in macrophages by suppressing nuclear factor-κB activation and BH_4 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.

roscovitine; nitric oxide; tetrahydrobiopterin; nuclear factor κB; macrophage

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 (BH_4) is an essential cofactor for all NOS isoforms and has been shown to be an important regulator of iNOS activity and iNOS-dependent •NO production (1, 4, 28). GTP cyclohydrolase-I (GCH-1) is the first step and the rate-limiting enzyme for BH_4 biosynthesis in the de novo pathway. GCH-1 transgenic mice showed significantly enhanced renal iNOS expression and •NO levels with LPS treatment (36). Inhibition of GCH-1 suppressed LPS-induced •NO production (15) and IFNγ-induced iNOS expression (5).

In our recent study on regulation of GCH-1 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 GCH-1 activity and BH_4 production (unpublished observations). Since T85 on GCH-1 has the consensus sequence for cyclin-dependent kinase 5 (CDK5), we originally hypothesized that inhibition of CDK5 may suppress GCH-1 phosphorylation and, accordingly, •NO production in macrophages. However, our results indicated that CDK5 inhibitor roscovitine directly inhibited GCH-1 expression (mRNA and protein) induced by LPS. Therefore, we hypothesize that roscovitine inhibits LPS-induced •NO production by inhibiting BH_4 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 GCH-1, production of •NO and BH_4, 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-amino-

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pyrimidin-4-yl)-sulfanilamide, and CDK2 inhibitor II (compound 3) were purchased from Calbiochem (San Diego, CA). Anti-GCH-1 antibody was generously provided by Dr. Zvonimir S. Katusic (29). Antibodies against phospho-ERK, phospho-p38, phospho-c-Jun NH_2-terminal kinase (JNK), phospho-P65, P65, phospho-IκB, and phospho-IκB kinase (IKK) were from Cell Signaling Technology (Boston, MA). Antibodies against iNOS and GAPDH were from Santa Cruz Biotechnology (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 BH_4 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% CO_2 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 prewarmed 1640 medium twice. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin. Rats used in this study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, by the National Research Council. The eluted macrophages were centrifuged for 10 min 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 2 h of being plated, the unattached cells were washed off by PBS, and the peritoneal macrophages were incubated with 1640 medium containing 10% FBS for all experiments.

RNA extraction and 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 real-time (RT)-PCR were synthesized by Operon Biotechnologies (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′-GAC AAA GCC AGA GTC CTT CA-3′ (forward) and 5′-TTG GAT GGT CTT GGT CCT TA-3′ (reverse); and the sequences for TNFα were 5′-AAA ATT CGA GTG ACA AGC CTG TAG-3′ (forward) and 5′- CCC TTG AAG AGA ACC TGG GAG TAG-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 (C_t) values between the target gene (GCT-1 or iNOS, etc.) and the reference gene (GAPDH) is defined as ΔC_t ($C_{t\ \text{target gene}} - C_{t\ \text{GADPH}}$), then mRNA expressions were calculated as $2^{\Delta C_t}$ and expressed by fold increase over the unstimulated macrophages ($2^{\Delta C\ \text{LPS}}/2^{\Delta C\ \text{no LPS}}$).

•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 a 96-well plate. After incubation with sulfanilamide solution (50 μl) for 10 min at room temperature, the medium was then mixed with NED solution (50 μl) for 10 min at room temperature. 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 NO_2^- in the samples was calculated from a sodium nitrite standard curve using freshly prepared NO_2^- standards (0–100 μM).

BH_4 and BH_2 assay. BH_4 and BH_2 were assayed by using HPLC with an electrochemical detector (ESA Biosciences CoulArray system model 542). Cell pellets were immediately lysed by passing a 28-gauge tuberculin syringe in 150 μl 50 mM phosphate buffer (pH 2.6) containing 0.2 mM diethylenetriaminepentaacetic acid and 1 mM 1,4-dithioerythritol (freshly added). Samples were centrifuged at 12,000 *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). Multichannel coulometric detection was set between 0–600 mV. One channel was set at −250 mV to verify the reversibility of BH_4 oxidative peak detection. Calibration curves were made by summation of the peak areas collected at 0 and 150 mV for BH_4 and 280 and 365 mV for BH_2. Intracellular concentrations were calculated using authentic BH_4 and BH_2 standards. BH_4 and BH_2 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 h. The blotted mem-

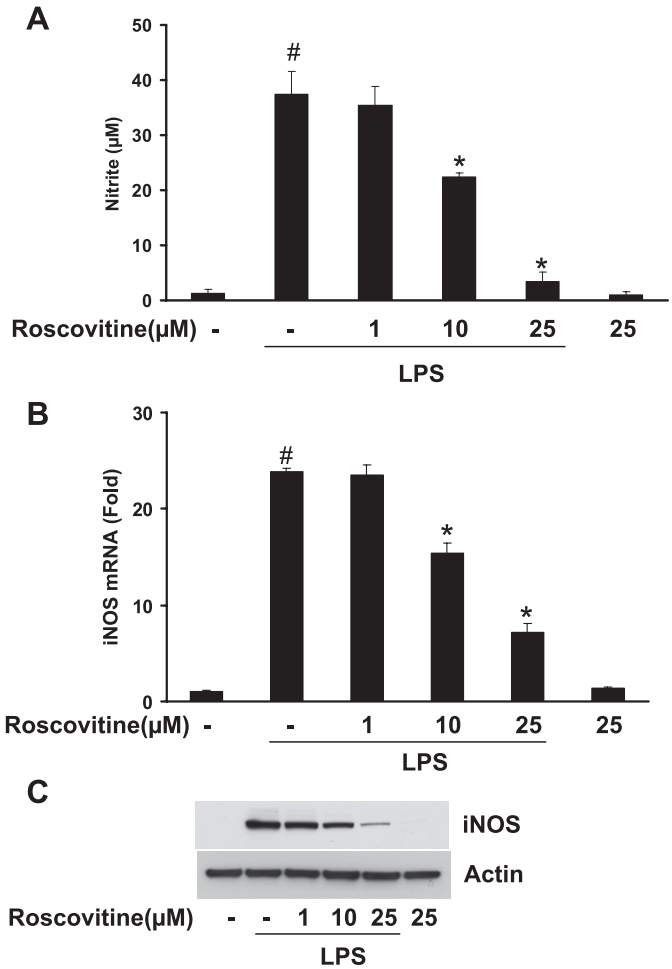

Fig. 1. Roscovitine inhibited inducible nitric oxide (NO) synthase (iNOS) expression and •NO production. Cells were pretreated with roscovitine at different concentrations for 30 min before stimulated with lipopolysaccharide (LPS, 2 μg/ml) for 12 h. Supernatant was collected for nitrite production (*A*)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

branes were visualized with Super Signal West Pico kit (Pierce Biotechnology).

Statistics. Data are expressed as means ± SE 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 generation induced by LPS. To determine the potential cell cytotoxicity of roscovitine, RAW 264.7 macrophages were treated with roscovitine at 1, 10, and 25 μM for 24 h, and cell viability was measured by MTT assay. As shown in Supplement Fig. IA, roscovitine at all concentrations did not 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 Fig. 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, 10, and 25 μM) for 30 min followed by stimulation with LPS (2 μg/ml) for 12 h. As expected, LPS significantly increased the NO_2^- 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 and 25 μM of roscovitine dose dependently inhibited •NO production from macrophages, whereas roscovitine at 25 μM alone had no effect (Fig. 1*A*). To determine whether roscovitine would also inhibit •NO production by LPS at a concentration comparable to physiological conditions, we treated the cells with LPS (100 ng/ml) for 12 h and determined •NO production. LPS at 100 ng/ml significantly increased •NO production (Supplement Fig. 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 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 reactive O_2 species (ROS) is another marker of LPS-induced proinflammatory response. Intriguingly, roscovitine also significantly inhibited LPS-induced ROS production in macrophages (Supplement Fig. IV).

Roscovitine inhibited LPS-induced NFκB activation. As shown in Fig. 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 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 (Fig. 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 (Fig. 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 TNF-α 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 TNF-α. We found that roscovitine dose dependently inhibited COX-2 protein expression at 10 and 25 μM. Roscovitine at 25 μM alone did not affect the COX-2 expression (Fig. 3*A*). RT-PCR results showed that LPS significantly increased IL-1β and IL-6 mRNA expressions. Roscovitine at 10 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 TNF-α induction by LPS (Fig. 3*D*). On the contrary, roscovi-

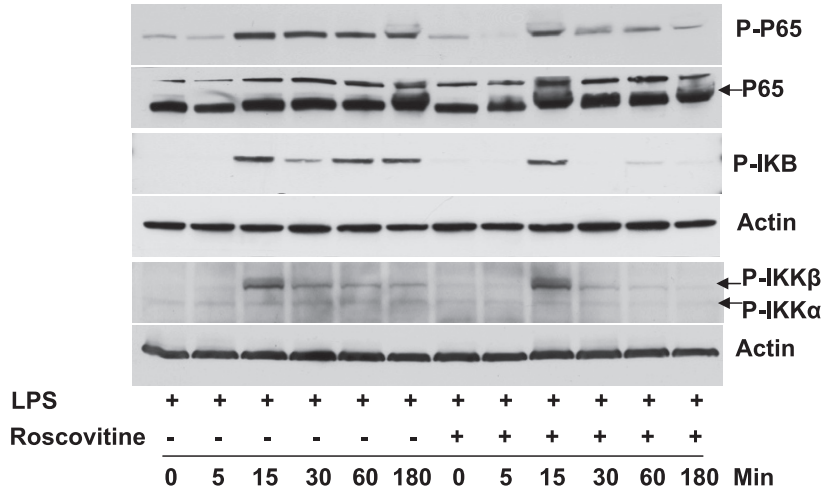

Fig. 2. Roscovitine inhibited the nuclear factor (NF)κB activation by LPS. Cells were pretreated with roscovitine (25 μM) for 30 min before incubated with LPS at different times as shown above. The immunoblot of cell lysate was probed with antibodies against phospho-IκB, phospho-p65, and phospho-IκB kinase (IKK) and reprobed with antibodies against p65 or actin. The results are representative of 4 blots.

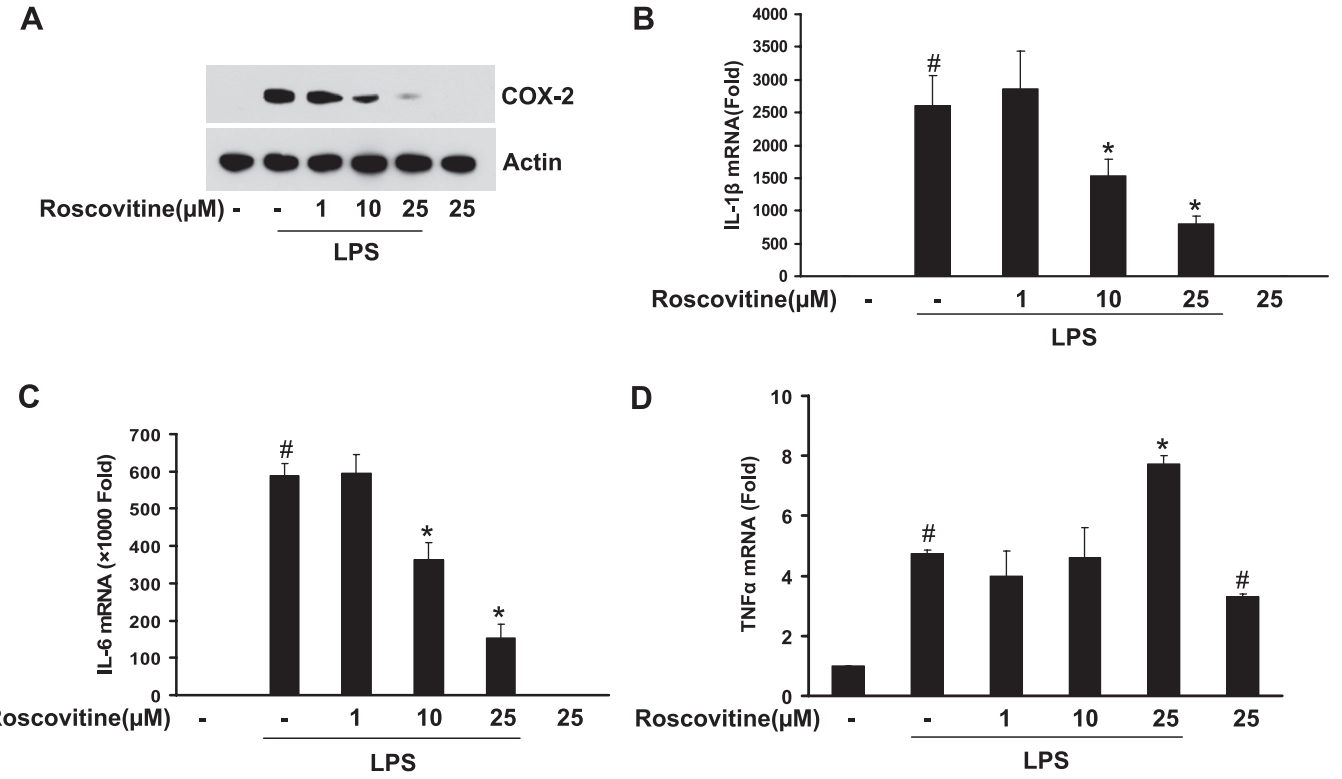

Fig. 3. Roscovitine inhibited cyclooxygenase (COX-2), IL-1β, and IL-6 induction by LPS. Cells were pretreated with roscovitine for 30 min followed by LPS stimulation for 12 h. *A*: cell proteins were immunoblotted with antibodies against COX-2 and actin, and the RNA was extracted to determine IL-1β (*B*)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

tine alone or with LPS further increased TNF-α 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 toward 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 and 60 min (Fig. 4). In the same trend, roscovitine enhanced the LPS-induced p38 phosphorylation at 15 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 BH_4 production and GCH-1 expression. After 12 h treatment, LPS increased BH_4 levels in the cells more than sixfold. Roscovitine (25 μM) strongly inhib-

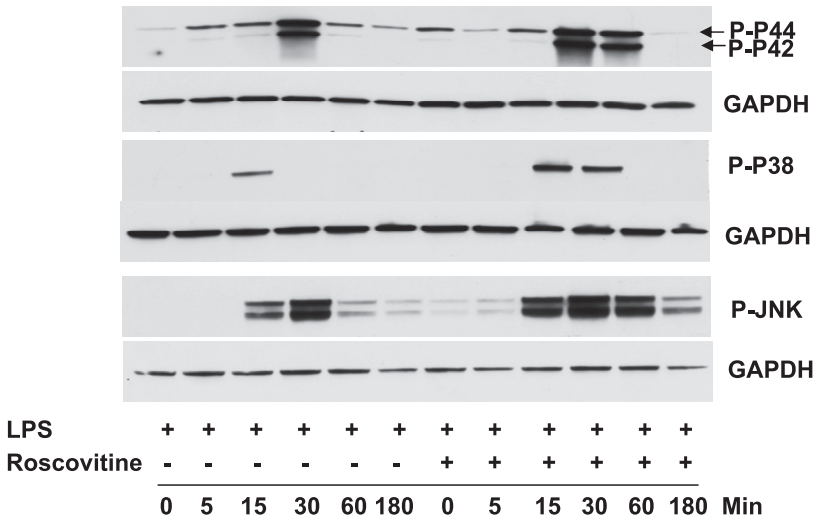

Fig. 4. Roscovitine did not inhibit mitogen-activated protein kinase (MAPK) activation by LPS. Cells were pretreated with roscovitine (25 μM) for 30 min before incubated with LPS at different times as shown. The immunoblot of cell lysate was probed with antibodies against phospho-ERK, phospho-JNK, and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

ited the LPS-induced BH_4 to a level even below the control levels; however, roscovitine alone had no effect on BH_4 production (Fig. 5). Interestingly, no significant difference was observed for BH_2 levels with roscovitine treatment, although roscovitine alone had a tendency to increase BH_2 levels. Therefore, roscovitine also significantly reduced the BH_4-to-BH_2 ratio, which was indicated to be just as important for NOS-coupled activity as total BH_4 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 by LPS (Fig. 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} (Fig. 7). CDK1 inhibitor did not inhibit •NO production at 10 μM but dose dependently inhibited •NO production between 25 and 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 Fig. II).

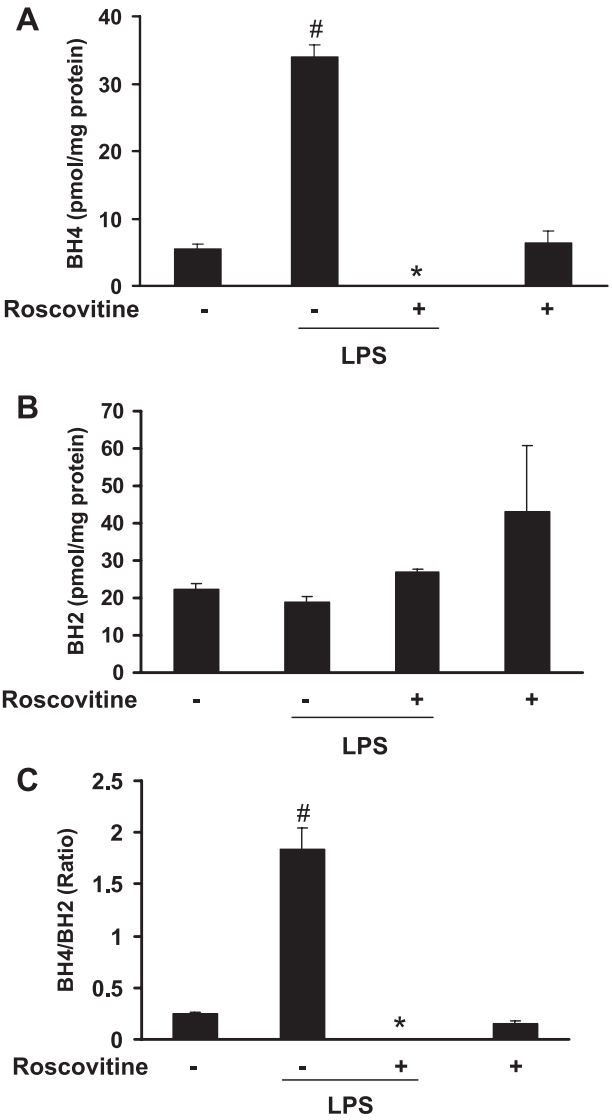

Fig. 5. Roscovitine inhibited tetrahydrobiopterin (BH_4) production by LPS. Cells were pretreated with roscovitine (25 μM) for 30 min before incubated with LPS for 12 h. Cells were lyzed in acidic buffer (see MATERIALS AND METHODS) and analyzed by HPLC for BH_4 (*A*) and 7,8-dihydrobiopterin (BH_2) (*B*) levels. The BH_4-to-BH_2 ratio was shown in *C*. The experiments were repeated 3 times. #$P < 0.01$ vs. control and *$P < 0.01$ vs. LPS alone.

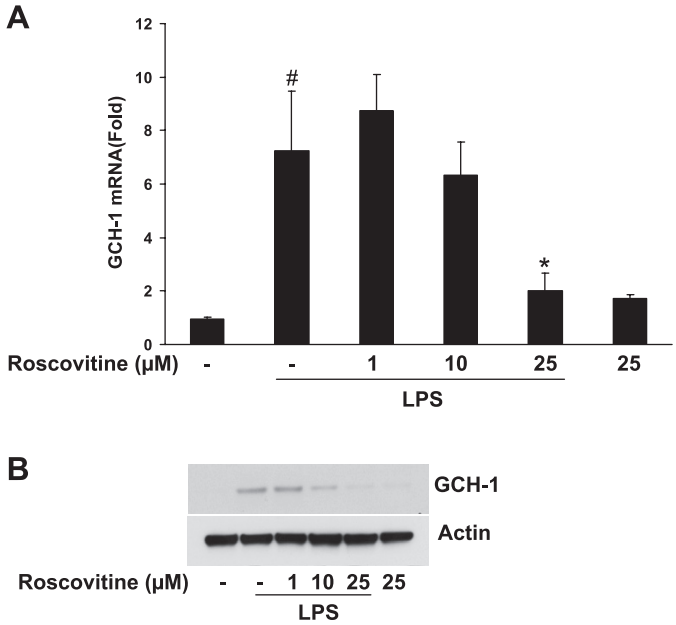

Fig. 6. Roscovitine inhibited GTP cyclohydrolase-1 (GCH-1) expression. Cells were pretreated with roscovitine for 30 min before stimulated with LPS for 12 h. Cells were lyzed for real-time PCR of GCH-1 mRNA expression (*A*) or immunoblot of GCH-1 protein expression (*B*) by probing against antibodies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

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 h) in peritoneal macrophages (Fig. 8, *A* and *B*). Moreover, roscovitine also obviously decreased the upregulation of GCH-1 by LPS, indicating that BH_4 synthesis is also inhibited by roscovitine (Fig. 8*C*). 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) (Fig. 8*D*).

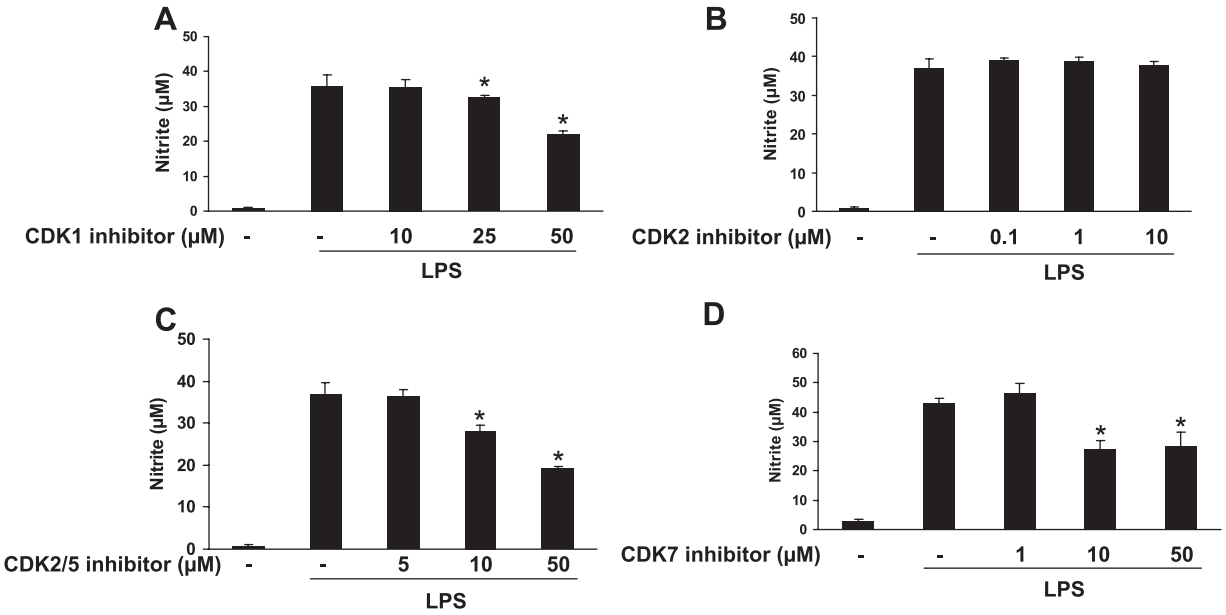

Fig. 7. Effects of CDK inhibitors on •NO production. Cells were pretreated with different CDK inhibitors at different concentrations as indicated for 30 min before stimulated with LPS for 12 h. Supernatant was collected for nitrite production. Results were from 3 separate experiments. *$P < 0.01$ vs.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

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 GCH-1-dependent BH_4 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 downregulate NFκB activation in response to TNF-α and interleukin 1 in A549 and 293 cells (11). During preparation of this paper, a study similar to ours showed that roscovitine downregulated 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 (Figs. 4 and 8). Furthermore, roscovitine was found to be able to abolish the generation of ROS acutely induced by LPS (Supplement Fig. IV). LPS activates NFκB by the canonical pathway; phosphorylation of IκB by IKK triggers the degradation of IκBα to activate NFκB p65 subunit (23). Because 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

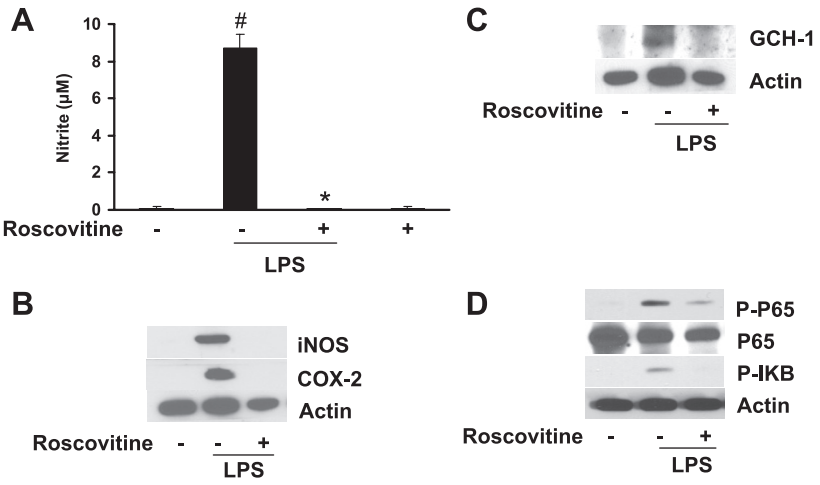

Fig. 8. Effects of roscovitine on inflammatory response, NFκB activation, and GCH-1 expression in primary macrophages. Rat peritoneal macrophages were pretreated with roscovitine (25 μM, 30 min) and stimulated with LPS (1 μg/ml) for 12 h. The supernatant was collected to assay nitrite concentrations (*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*: macrophages were stimulated with LPS for 30 min to determine the phosphorylation of P65 and IκB by Western blot analysis.

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 upregulates the expression of COX-2, IL-6, IL-1β, and TNF-α, 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 TNF-α expression (Fig. 6). Because inhibition of ERK and JNK also block TNF-α production (10, 25), we speculate that increased TNF-α expression is due to roscovitine activating MAPK (Fig. 4), which has been shown in keratinocytes (2) and human colon cancer cell lines (37, 38). The increased TNF-α may also contribute to the proapoptotic effect of roscovitine on inflammatory cells (Supplement Fig. 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 BH_4, which stabilizes dimer formation (28). In macrophages, a deficiency in BH_4 impaired •NO production after LPS stimulation (31). In our study, roscovitine significantly inhibited LPS-induced BH_4 production and decreased the BH_4-to-BH_2 ratios. At 25 μM of roscovitine, although iNOS expression was inhibited it was still approximately fourfold higher than the basal levels. Yet, LPS-induced •NO production was reduced to unstimulated levels. This demonstrates just how important BH_4 is for optimal iNOS activity. As the major rate-limiting enzyme in BH_4 biosynthesis, GCH-1 could be regulated in cells and tissues at the level of transcription and posttranslational modification (39). Inhibition of phosphoinositide 3-kinase (PI3K) counteracted LPS-induced GCH-1 expression in macrophages (27). In PI3K-deficient macrophages, the induction of GCH-1 and biosynthesis of BH_4 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 GCH-1 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 in vitro and 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 BH_4 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.

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REFERENCES

1. **Andrew PJ, Mayer B.** Enzymatic function of nitric oxide synthases. *Cardiovasc Res* 43: 521–531, 1999.
2. **Atanasova G, Jans R, Zhelev N, Mitev V, Poumay Y.** Effects of the cyclin-dependent kinase inhibitor CYC202 (R-roscovitine) on the physiology of cultured human keratinocytes. *Biochem Pharmacol* 70: 824–836, 2005.
3. **Bach S, Knockaert M, Reinhardt J, Lozach O, Schmitt S, Baratte B, Koken M, Coburn SP, Tang L, Jiang T, Liang DC, Galons H, Dierick JF, Pinna LA, Meggio F, Totzke F, Schachtele C, Lerman AS, Carnero A, Wan Y, Gray N, Meijer L.** Roscovitine targets, protein kinases and pyridoxal kinase. *J Biol Chem* 280: 31208–31219, 2005.
4. **Baek KJ, Thiel BA, Lucas S, Stuehr DJ.** Macrophage nitric oxide synthase subunits. Purification, characterization, and role of prosthetic groups and substrate in regulating their association into a dimeric enzyme. *J Biol Chem* 268: 21120–21129, 1993.
5. **Bogdan C, Werner E, Stenger S, Wachter H, Rollinghoff M, and Werner-Felmayer G.** 2,4-Diamino-6-hydroxypyrimidine, an inhibitor of tetrahydrobiopterin synthesis, downregulates the expression of iNOS protein and mRNA in primary murine macrophages. *FEBS Lett* 363: 69–74, 1995.
6. **Bogush A, Pedrini S, Pelta-Heller J, Chan T, Yang Q, Mao Z, Sluzas E, Gieringer T, Ehrlich ME.** AKT and CDK5/p35 mediate brain-derived neurotrophic factor induction of DARPP-32 in medium size spiny neurons in vitro. *J Biol Chem* 282: 7352–7359, 2007.
7. **Caivano M.** Role of MAP kinase cascades in inducing arginine transporters and nitric oxide synthetase in RAW264 macrophages. *FEBS Lett* 429: 249–253, 1998.
8. **Chen CC, Wang JK.** p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages. *Mol Pharmacol* 55: 481–488, 1999.
9. **Crabtree MJ, Smith CL, Lam G, Goligorsky MS, Gross SS.** Ratio of 5,6,7,8-tetrahydrobiopterin to 7,8-dihydrobiopterin in endothelial cells determines glucose-elicited changes in NO vs. superoxide production by eNOS. *Am J Physiol Heart Circ Physiol* 294: H1530–H1540, 2008.
10. **Debbas V, Arai RJ, Ferderbar S, Schindler F, Stern A, Monteiro HP.** Regulation of p21Waf1 expression and TNFalpha biosynthesis by glutathione modulators in PMA induced-THP1 differentiation: involvement of JNK and ERK pathways. *Biochem Biophys Res Commun* 363: 965–970, 2007.
11. **Dey A, Wong ET, Cheok CF, Tergaonkar V, Lane DP.** R-Roscovitine simultaneously targets both the p53 and NF-kappaB pathways and causes potentiation of apoptosis: implications in cancer therapy. *Cell Death Differ* 15: 263–273, 2008.
12. **Du J, Xu H, Wei N, Wakim B, Halligan B, Pritchard KA Jr, Shi Y.** Identification of proteins interacting with GTP cyclohydrolase I. *Biochem Biophys Res Commun* 285: 143–147, 2009.
13. **Festa M, Petrella A, Alfano S, Parente L.** R-roscovitine sensitizes anaplastic thyroid carcinoma cells to TRAIL-induced apoptosis via regulation of IKK/NF-kappaB pathway. *Int J Cancer* 124: 2728–2736, 2009.

14. **Fu AK, Fu WY, Ng AK, Chien WW, Ng YP, Wang JH, Ip NY.** Cyclin-dependent kinase 5 phosphorylates signal transducer and activator of transcription 3 and regulates its transcriptional activity. *Proc Natl Acad Sci USA* 101: 6728–6733, 2004.
15. **Geller DA, Di Silvio M, Billiar TR, Hatakeyama K.** GTP cyclohydrolase I is coinduced in hepatocytes stimulated to produce nitric oxide. *Biochem Biophys Res Commun* 276: 633–641, 2000.
16. **Hacker H, Karin M.** Regulation and function of IKK and IKK-related kinases. *Sci STKE* 357: re13, 2006.
17. **Huang A, Zhang YY, Chen K, Hatakeyama K, Keaney JF Jr.** Cytokine-stimulated GTP cyclohydrolase I expression in endothelial cells requires coordinated activation of nuclear factor-kappaB and Stat1/Stat3. *Circ Res* 96: 164–171, 2005.
18. **Jhou RS, Sun KH, Sun GH, Wang HH, Chang CI, Huang HC, Lu SY, Tang SJ.** Inhibition of cyclin-dependent kinases by olomoucine and roscovitine reduces lipopolysaccharide-induced inflammatory responses via down-regulation of nuclear factor kappaB. *Cell Prolif* 2009.
19. **Kleinert H, Pautz A, Linker K, Schwarz PM.** Regulation of the expression of inducible nitric oxide synthase. *Eur J Pharmacol* 500: 255–266, 2004.
20. **Korhonen R, Lahti A, Kankaanranta H, Moilanen E.** Nitric oxide production and signaling in inflammation. *Curr Drug Targets Inflamm Allergy* 4: 471–479, 2005.
21. **Lalioti V, Muruais G, Dinarina A, van Damme J, Vandekerckhove J, Sandoval IV.** The atypical kinase Cdk5 is activated by insulin, regulates the association between GLUT4 and E-Syt1, and modulates glucose transport in 3T3–L1 adipocytes. *Proc Natl Acad Sci USA* 2009.
22. **Liew FY.** Regulation of nitric oxide synthesis in infectious and autoimmune diseases. *Immunol Lett* 43: 95–98, 1994.
23. **Liu J, Lin A.** Wiring the cell signaling circuitry by the NF-kappa B and JNK1 crosstalk and its applications in human diseases. *Oncogene* 26: 3267–3278, 2007.
24. **Mapelli M, Massimiliano L, Crovace C, Seeliger MA, Tsai LH, Meijer L, Musacchio A.** Mechanism of CDK5/p25 binding by CDK inhibitors. *J Med Chem* 48: 671–679, 2005.
25. **Marantos C, Mukaro V, Ferrante J, Hii C, Ferrante A.** Inhibition of the lipopolysaccharide-induced stimulation of the members of the MAPK family in human monocytes/macrophages by 4-hydroxynonenal, a product of oxidized omega-6 fatty acids. *Am J Pathol* 173: 1057–1066, 2008.
26. **Noble ME, Endicott JA, Johnson LN.** Protein kinase inhibitors: insights into drug design from structure. *Science* 303: 1800–1805, 2004.
27. **Ota A, Kaneko YS, Mori K, Nakashima A, Nagatsu I, Nagatsu T.** Effect of peripherally administered lipopolysaccharide (LPS) on GTP cyclohydrolase I, tetrahydrobiopterin and norepinephrine in the locus coeruleus in mice. *Stress* 10: 131–136, 2007.
28. **Panda K, Rosenfeld RJ, Ghosh S, Meade AL, Getzoff ED, Stuehr DJ.** Distinct dimer interaction and regulation in nitric-oxide synthase types I, II, and III. *J Biol Chem* 277: 31020–31030, 2002.
29. **Peterson TE, d'Uscio LV, Cao S, Wang XL, Katusic ZS.** Guanosine triphosphate cyclohydrolase I expression and enzymatic activity are present in caveolae of endothelial cells. *Hypertension* 53: 189–195, 2009.
30. **Rossi AG, Sawatzky DA, Walker A, Ward C, Sheldrake TA, Riley NA, Caldicott A, Martinez-Losa M, Walker TR, Duffin R, Gray M, Crescenzi E, Martin MC, Brady HJ, Savill JS, Dransfield I, Haslett C.** Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. *Nat Med* 12: 1056–1064, 2006.
31. **Sakai K, Suzuki H, Oda H, Akaike T, Azuma Y, Murakami T, Sugi K, Ito T, Ichinose H, Koyasu S, Shirai M.** Phosphoinositide 3-kinase in nitric oxide synthesis in macrophage: critical dimerization of inducible nitric-oxide synthase. *J Biol Chem* 281: 17736–17742, 2006.
32. **Senderowicz AM.** Novel small molecule cyclin-dependent kinases modulators in human clinical trials. *Cancer Biol Ther* 2: S84–S95, 2003.
33. **Shi Y, Pritchard KA Jr, Holman P, Rafiee P, Griffith OW, Kalyanaraman B, and Baker JE.** Chronic myocardial hypoxia increases nitric oxide synthase and decreases caveolin-3. *Free Radic Biol Med* 29: 695–703, 2000.
34. **Vasquez-Vivar J, Martasek P, Whitsett J, Joseph J, Kalyanaraman B.** The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. *Biochem J* 362: 733–739, 2002.
35. **Wang CX, Song JH, Song DK, Yong VW, Shuaib A, Hao C.** Cyclin-dependent kinase-5 prevents neuronal apoptosis through ERK-mediated upregulation of Bcl-2. *Cell Death Differ* 13: 1203–1212, 2006.
36. **Wang W, Zolty E, Falk S, Summer S, Zhou Z, Gengaro P, Faubel S, Alp N, Channon K, Schrier R.** Endotoxemia-related acute kidney injury in transgenic mice with endothelial overexpression of GTP cyclohydrolase-1. *Am J Physiol Renal Physiol* 294: F571–F576, 2008.
37. **Whittaker SR, Te Poele RH, Chan F, Linardopoulos S, Walton MI, Garrett MD, Workman P.** The cyclin-dependent kinase inhibitor seliciclib (R-roscovitine; CYC202) decreases the expression of mitotic control genes and prevents entry into mitosis. *Cell Cycle* 6: 3114–3131, 2007.
38. **Whittaker SR, Walton MI, Garrett MD, Workman P.** The Cyclin-dependent kinase inhibitor CYC202 (R-roscovitine) inhibits retinoblastoma protein phosphorylation, causes loss of Cyclin D1, and activates the mitogen-activated protein kinase pathway. *Cancer Res* 64: 262–272, 2004.
39. **Widder JD, Chen W, Li L, Dikalov S, Thony B, Hatakeyama K, Harrison DG.** Regulation of tetrahydrobiopterin biosynthesis by shear stress. *Circ Res* 101: 830–838, 2007.