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Mechanisms Underlying Nonsteroidal Anti-Inflammatory Drug-Induced p27^{Kip1} Expression

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ABSTRACT

We demonstrated previously that nonsteroidal anti-inflammatory drugs (NSAIDs) increased p27^{Kip1} by inhibiting protein degradation to suppress the proliferation of human lung cancer cells. In this study, we elucidate the molecular mechanism by which NSAIDs modulate p27^{Kip1} proteolysis. Immunoblotting and in vitro ubiquitination assays indicated that the expression of Cul1 and Skp2 and ubiquitination activity toward p27^{Kip1} were not regulated by NSAIDs. On the contrary, we found that NSAIDs inhibited proteasome activity to increase p27^{Kip1} protein levels. NSAIDs suppressed the expression of chymotrypsin-like catalytic subunits (β5, LMP7, and LMP2), but did not directly block enzymatic activity, to inhibit proteasome activity. Reverse transcriptase-competitive polymerase chain reaction and promoter activity assays showed that this inhibition occurred at the transcriptional level. In vitro degradation experiments showed that p27^{Kip1} degradation was inhibited by NS398, and the addition of purified 26S proteasome reversed this inhibitory effect. Collectively, our results revealed the mechanism by which NSAIDs modulate p27^{Kip1} protein degradation and suggest that NSAIDs are a novel class of proteasome inhibitors.

Emerging evidence demonstrates that nonsteroidal anti-inflammatory drugs (NSAIDs) exhibit a significant antiproliferative effect on a variety of cancer cells (Goldberg et al., 1996; Sheng et al., 1997; Thompson et al., 1997; Molina et al., 1999). Additionally, epidemiological studies also indicate that NSAID use is associated with a reduced risk of cancer development (Gupta and DuBois, 1998). However, the mechanisms by which NSAIDs inhibit tumor growth are not well-defined. Some NSAIDs may induce apoptosis in cancer cells (Shiff et al., 1995; Elder et al., 2000; Rahman et al., 2000), and the apoptosis-inducing activity of these drugs is linked with suppression of the antiapoptotic bcl-2 gene or with the induction of ceramide, a well-known mediator of apoptosis, in cancer cells (Chan et al., 1998; Liu et al., 1998). However, most NSAIDs at pharmacological doses induce growth inhibition rather than apoptosis in cancer cells. At present, the molecular basis for this inhibitory action is largely unknown.

The progression of the mammalian cell cycle is controlled by three major gene families, including cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CDKIs). Cyclins and CDKs are positive cell-cycle regulators, and the up-regulation of these two gene families is frequently found in human cancers (Sherr, 1995). Conversely, CDKIs are negative cell-cycle regulators, and the down-regulation of these inhibitory proteins is a general phenomenon observed in human cancers (Sherr and Robert, 1995). We showed previously that an NSAID, NS398, may suppress the growth of human lung cancer cells by up-regulating the expression of p27^{Kip1}, a typical CDKI (Hung et al., 2000). Additionally, we showed that NS398 increased the intracellular level of p27^{Kip1} via inhibition of protein degradation. Two other recent studies showed that aspirin, a generally used NSAID, also increased intracellular p27^{Kip1} protein levels to suppress the proliferation of vascular smooth muscle cells and colon cancer cells (Marra et al., 2000; Kralj et al., 2001). These results suggest that the up-regulation of p27^{Kip1} is one of the mechanisms by which NSAIDs inhibit cell growth.

The intracellular level of p27^{Kip1} is controlled by post-translational modification. Recent works showed that this CDKI is degraded via the ubiquitin/proteasome pathway. After being phosphorylated at T187 by cyclin E/CDK2 complex, p27^{Kip1} is ubiquitinated by the sequential action of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) (Hershko and Ciechanover, 1998; Kornitzer and Ciechanover, 2000). Polyubiquitinated p27^{Kip1} protein is subjected to degradation by the 26S pro-

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; NS398, *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-polymerase chain reaction; PCR, polymerase chain reaction; bp, base pair(s); Suc-LLVY-AMC, *N*-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal.

teasome (Reits et al., 1997; Ciechanover, 1998). In this study, we investigated the mechanism by which NSAIDs modulate $p27^{Kip1}$ protein degradation.

Materials and Methods

Cells and Reagents. A549 human lung cancer cells were maintained in Dulbecco's modified Eagle's medium and Ham's F12 nutrition mixture with 10% fetal calf serum and antibiotics. NS398, 20S, and 26S proteasome and methylubiquitin were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). The LMP2 promoter-luciferase construct was kindly provided by Dr. Jenny J. P. Ting (University of North Carolina, Chapel Hill, NC). Luciferase and β-galactosidase assay systems were obtained from Promega (Madison, WI). The His6-tagged human $p27^{Kip1}$ expression vector was a gift from Dr. Michele Pagano (Department of Pathology, NYU School of Medicine, New York, NY).

Immunological Reagents and Procedures. For immunoblotting, cells were treated with vehicle (0.5% dimethyl sulfoxide) or drugs (indomethacin or NS398) for different times and harvested with a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 2 μg/ml pepstatin A, and 2 μg/ml leupeptin). The determination of protein concentration, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were performed as described previously (Hung et al., 2000). Antibodies used in this study included Cul1, Skp2, $p27^{Kip1}$, and His6-tagged protein from Santa Cruz Biochemicals (Santa Cruz, CA); actin was from Chemicon International (Temecula, CA); and LMP7 and LMP2 were purchased from Affiniti Research Products (Mamhead, United Kingdom). Polyclonal antibody against human proteasome β5 subunit was provided by Dr. K. B. Hendil (August Krogh Institute, University of Copenhagen, Copenhagen, Denmark).

Preparation of Cell Extracts for In Vitro Ubiquitination Assay. Control or drug-treated cells were washed with phosphate-buffered saline and harvested in a lysis buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 25% glycerol, 1 mM EDTA, 2.5 mM dithiothreitol, and protease inhibitors). Cells were frozen and thawed three times, and the lysates were subjected to centrifugation at 10,000*g* for 20 min at 4°C. The supernatants were frozen at −70°C until assays were performed.

Expression of His6-Tagged Human $p27^{Kip1}$. His6-tagged human $p27^{Kip1}$ vector was expressed in *Escherichia coli,* and expressed proteins were purified by nickel-agarose chromatography according to the manufacturer's instructions (QIAGEN, Valencia, CA).

In Vitro Ubiquitination Assays. His6-tagged human $p27^{Kip1}$ protein was incubated with the cell extracts (containing equal amount of cellular proteins) in the presence of an ATP-generating system (50 mM Tris, pH 8.3, 5 mM $MgCl_2$, 5 mM ATP, 10 mM creatine phosphate, and 0.2 unit/ml creatine kinase) together with 2 mM dithiothreitol, 1 mg/ml methylubiquitin, and protease inhibitors. Reactions were carried out at 37°C for 30 min and were terminated by adding SDS sample buffer. Reaction mixtures were subjected to SDS-PAGE and probed with anti–His6-tagged protein antibody.

Assays for Proteasome Activity in Cells. To investigate the effect of NSAIDs on intracellular proteasome activity, cell lysates were prepared, and fluorogenic peptide substrate assays were performed according to the procedures described by Glas et al. (1998). In brief, control or drug-treated cells were broken by glass beads (<106 μm, acid-washed; Sigma Chemical, St. Louis, MO) in a homogenization buffer (50 mM Tris, pH 7.4, 1 mM dithiothreitol, 5 mM $MgCl_2$, 2 mM ATP, and 250 mM sucrose). Cells were then vortexed for 1 min. Beads and cell debris were removed by centrifugation at 1,000*g* for 5 min, followed by 10,000*g* for 20 min at 4°C. The supernatant was collected, and the protein concentration was determined. For an assay of chymotrypsin-like activity of the proteasome, the fluorogenic substrate Suc-LLVY-AMC (Biomol) was used. Total cell lysates (10 μg) were diluted to 100 μl in a reaction buffer (50 mM Tris, pH 7.4, 1 mM dithiothreitol, 5 mM $MgCl_2$, and 2 mM ATP). Substrates (100 μM) were added to samples and incubated at 37°C for 45 min. The reaction was stopped with 1% SDS solution, and the intensity of fluorescence was measured by a fluorescent spectrophotometer (BMG Labtechnologies, Offenburg, Germany).

In Vitro Proteasome Activity Assay. To determine whether NS398 and indomethacin are direct inhibitors of proteasome, an in vitro proteasome activity assay was performed. Purified 20S proteasome (0.5 μg) was diluted to 100 μl in a reaction buffer (50 mM Tris, pH 7.4, 1 mM dithiothreitol, 5 mM $MgCl_2$, and 2 mM ATP). NS398, indomethacin, or the proteasome inhibitor MG132 was added into the reaction buffer. Fluorogenic substrate Suc-LLVY-AMC (100 μM) was then added to samples and incubated at 37°C for 45 min. The reaction was terminated, and the intensity of fluorescence was measured as described above.

Construction of Competitor Templates. A competitive reverse transcriptase-polymerase chain reaction (RT-PCR) construct (mimic) for LMP2 was synthesized using the sense primer 5′-CCTTGCAGGGATGCTGCGGATACTTTCGGCAGCACCTC-3′, in which nucleotides 1 through 17 of LMP2 (GenBank accession number U01025) were attached to nucleotides 35 through 54 of the proteasome α6 subunit (GenBank accession number XM006212), and the antisense primer 5′-GGGAAGGTTCACTCATCACCATTGGTTCATCAGC CTTT-3′, in which nucleotides 630 through 645 of LMP2 were attached to nucleotides 868 through 887 of proteasome α6 subunit. Total RNA was isolated, and RT-PCR was performed as described previously (Lee et al., 2000). The PCR reaction mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 mM dNTP, 600 nM primers, and 3 units of HotStarTaq DNA polymerase, and the condition for PCR was 30 cycles of denaturation (94°C/1 min), annealing (60°C/45 s), extension (72°C/1 min), and 1 cycle of final extension (72°C/10 min).

The PCR products (888 bp) were electrophoresed on 1.2% agarose gels and purified using GenElute agarose spin columns according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). Similar procedures were used for the construction of LMP7 and proteasome β5 subunit mimics. The primer sequences used for LMP7 (GenBank accession number NM-004159) were 5′-GTGATGCTC ATAGGAACCGATACTTTCGGCAGCACCTC-3′ and 5′-GCCCCACCACCATTACCATTGGTTCATCAGCCT TT-3′; for the proteasome β5 subunit (GenBank accession number D29011), the sequences used were 5′-GACTTGGGGGT CGTGCAGATACTTTCGGCAGCACCTC-3′ and 5′-CACCTCTGCAG CAGCTCACCATTGGTTCATCAG CCTTT-3′.

Competitive PCR. Cells were treated with vehicle or drugs for 24 h; then total RNA was isolated, and reverse transcription was performed as described previously (Lee et al., 2000). PCR was carried out in a reaction buffer of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 mM dNTP, 600 nM primers, and 3 units of HotStarTaq DNA polymerase. The reverse-transcribed cDNA samples and various amounts of mimics were added to the reaction mixture and coamplified for 30 cycles of denaturation (94°C/1 min), annealing (60°C/1 min), extension (72°C/1 min), and 1 cycle of final extension (72°C/10 min). The primer sequences used for LMP2 were 5′-CCTTGCAGGGATGCTGCG-3′ and 5′-GGG AAGGTTCACTCATCA-3′; for LMP7, the sequences used were 5′-GTGATGCTCATAGGAACC-3′ and 5′-GCCCCACCACCATTA-3′; and for proteasome β5 subunit, the sequences were 5′-GACTTGGGGGTCGTGCA-3′ and 5′-CACCTCTGCAGCAGCTCA-3′. PCR products (10 μl) were separated by electrophoresis on 1.2% agarose gels and stained with ethidium bromide, and the intensity of the signals was analyzed by a densitometer.

Promoter Activity Assays. Cells were plated onto six-well plates at a density of 200,000 cells/well and grown overnight. Cells were then cotransfected with 2 μg of LMP2 promoter-luciferase construct and 2 μg of pCMV–β-galactosidase plasmid by the LipofectAMINE method (Invitrogen, Carslbad, CA) as described previ-

ously (Hung et al., 2000). Cells were incubated in the absence or presence of NSAIDs for 24 h, and luciferase activity was investigated by using the luciferase and β-galactosidase assay system (Promega). Luciferase activity was normalized with β-galactosidase activity and expressed as a percentage of the control cells. Results shown are from three independent experiments of duplicate samples.

In Vitro Degradation Assays. Cellular proteins were extracted according to the procedure described by Loda et al. (1997). Control or drug-treated cells were suspended in ice-cold double-distilled water. The samples were frozen and thawed three times, and the lysates were centrifuged at 15,000*g* for 30 min. The supernatants were collected for analysis. The total cell extracts prepared by this method have been shown to preserve ubiquitinating enzyme activity (Loda et al., 1997). Cell lysates (10 μg) were diluted to 100 μl in a reaction buffer (50 mM Tris, pH 7.4, 1 mM dithiothreitol, 5 mM $MgCl_2$, and 2 mM ATP), and 0.5 μg of His6-tagged $p27^{Kip1}$ was added. The reactions were carried out at 37°C for different times and were terminated by adding SDS sample buffer. Each reaction mixture was subjected to SDS-PAGE and probed with anti-His6 antibody to monitor the His6-tagged $p27^{Kip1}$ protein levels in the mixture. In some experiments, purified 26S proteasome (0.5 μg) was added exogenously to the reaction buffer.

Results

Expression of Cul1 and Skp2 and Ubiquitination of $p27^{Kip1}$ Were Not Affected by NSAIDs. Because our previous work indicated that NS398 up-regulated $p27^{Kip1}$ protein levels via inhibition of degradation, a direct prediction is that ubiquitination of $p27^{Kip1}$ may be affected by this drug. Ubiquitination of $p27^{Kip1}$ is catalyzed by the ubiquitin ligase $SCFp45^{Skp2}$. This ligase comprises Skp1, Cul1, Skp2 (an F-box protein), Roc1/Rbx1, and the recently identified Cks1 (Ganoth et al., 2001; Spruck et al., 2001). Among these components, the expression of Cul1 and Skp2 was found to be regulated by different extracellular stimuli (O'Hagan et al., 2000; Mamillapalli et al., 2001). Therefore, we tested whether NS398 and indomethacin may affect the expression of these two proteins. Our results showed that intracellular protein levels of Cul1 and Skp2 were not significantly regulated by these two NSAIDs (Fig. 1A). To further confirm these results, we performed an in vitro ubiquitination assay to clarify whether these two NSAIDs may disturb the ubiquitination of $p27^{Kip1}$. We used methylubiquitin in our experiments because it terminates the formation of polyubiquitin chains and thus causes the accumulation of easily detectable, discrete, low-molecular-mass (usually monoubiquitinated) derivatives rather than a "smear" of polyubiquitinated $p27^{Kip1}$. Our results demonstrated that monoubiquitinated $p27^{Kip1}$ was clearly detected in the immunoblots and that ubiquitination activity toward $p27^{Kip1}$ in A549 lung cancer cells was not affected by NS398 and indomethacin (Fig. 1B).

NSAIDs Inhibit Proteasome Activity in A549 Cells. Because NS398 and indomethacin did not inhibit $p27^{Kip1}$ ubiquitination, we next examined whether these two NSAIDs suppressed proteasome activity to increase intracellular $p27^{Kip1}$ protein levels. We used two approaches to address this question. First, it has been demonstrated that significant accumulation of ubiquitinated proteins can be detected easily in cells incubated with proteasome inhibitors. Therefore, we treated A549 cells with NS398, indomethacin, or MG132, a proteasome inhibitor, and investigated the change of protein ubiquitination by immunoblotting. Indeed, we found that the accumulation of ubiquitinated proteins was observed in NS398- or indomethacin-treated cells (Fig. 2). The pattern of protein accumulation in cells incubated with NSAIDs was similar to that of MG132-treated cells. Second, we examined the effect of NSAIDs on cellular proteasome activity. Cells were treated with vehicle or NSAIDs for 24 h, and cell lysates were harvested for analysis. A fluorogenic substrate was used to examine the chymotrypsin-like activity of proteasomes. As shown in Fig. 3, our results indicated that NS398 and indomethacin suppressed intracellular proteasome activity in a dose-dependent manner. However, the addition of prostaglandin E_2 (1 μM) could not significantly counteract the inhibitory effect of NS398 and indomethacin. These results suggest that the suppression of proteasome activity by NSAIDs may not be mediated via the inhibition of cyclooxygenase activity. We next investigated whether NS398 and indomethacin are direct enzymatic inhibitors for proteasomes. Purified 20S proteasomes were incubated with these two NSAIDs, and enzymatic activity was assayed. Our results indicated that NSAIDs could not directly inhibit the enzymatic activity of proteasomes (Fig. 4). This was not attributable to failures in the experiments, because MG132 effectively suppressed 20S proteasome activity under the same experimental conditions. Therefore, the

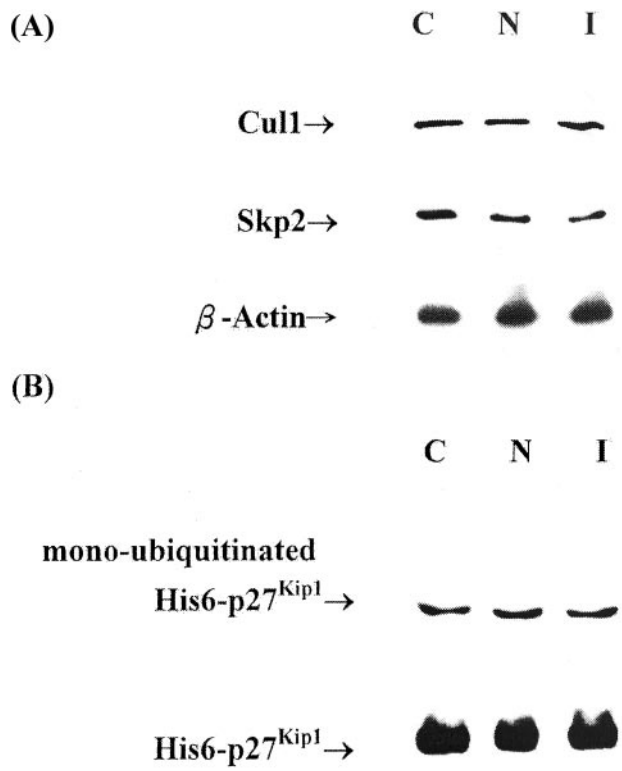

Fig. 1. Effect of NSAIDs on $p27^{Kip1}$ ubiquitination. A549 cells were treated with vehicle (C), 100 μM NS398 (N), or 100 μM indomethacin (I) for 24 h. A, cells were harvested in a lysis buffer, and Western blot analysis was performed to investigate the protein level of Cul1 and Skp2. β-actin was used as an internal control. B, cells were harvested in a buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 25% glycerol, 1 mM EDTA, 2.5 mM dithiothreitol, and protease inhibitors) and were frozen and thawed three times to lyse the cells. His6-tagged human $p27^{Kip1}$ protein was incubated with the cell lysates (containing equal amount of cellular proteins) in the presence of an ATP-generating system together with 2 mM dithiothreitol, 1 mg/ml methylubiquitin, and protease inhibitors. Reactions were carried out at 37°C for 30 min and terminated by adding SDS sample buffer. Reaction mixtures were subjected to SDS-PAGE and probed with anti–His6-tagged protein antibody.

suppression of intracellular proteasome activity by NSAIDs was not mediated via direct inhibition of enzymatic activity.

Suppression of Intracellular Proteasome Activity by NSAIDs Is Correlated with the Down-Regulation of Catalytic Subunits of Proteasome. Because NSAIDs could not inhibit proteasome activity directly, we speculated that NSAIDs might affect the expression and assembly of proteasome catalytic subunits in cells. Therefore, we tested the effect of NSAIDs on the expression of chymotrypsin-like catalytic subunits, including β5, LMP7, and LMP2, of standard proteasome or immunoproteasome. We developed an RT-competitive PCR assay in which the target (LMP2, LMP7, and β5 subunit) cDNA samples obtained after reverse transcription from control or drug-treated cells were coamplified in the same reaction tube in the presence of known amounts of competitor DNAs (mimics). The competitor and target DNAs use the same PCR primers but yield PCR products with different sizes. The PCR products (646 bp for LMP2 and 888 bp for mimic) from a typical competitive PCR assay were separated on agarose gel and visualized by ethidium bromide staining (Fig. 5A).

The amount of LMP2 was calculated to be 10,994 ± 2023 copies/μg of total RNA in control cells. Conversely, the amount of LMP2 was calculated to be 1983 ± 186 and 2,275 ± 372 copies/μg of total RNA in NS398- and indomethacin-treated cells, respectively. Thus, NSAIDs inhibited LMP2 expression by approximately 80% (Fig. 5B). Similarly, the expression of LMP7 and the β5 subunit were suppressed by NSAIDs (Fig. 5B). In accordance with the RT-PCR results, our data showed that NSAIDs also attenuated protein levels of these subunits in A549 cells (Fig. 5C). We next tested whether NSAIDs may directly inhibit gene transcription of these subunits. Because we did not have the promoter of the β5 and LMP7 genes, we first studied the effect of NSAIDs on the LMP2 promoter. As shown in Fig. 6, our results showed that NSAIDs inhibited LMP2 promoter activity in a dose-dependent manner. Additionally, prostaglandin E_2 could not reverse the inhibitory effect of NS398 and indomethacin. Thus, the inhibition of LMP2 by NSAIDs is not mediated via the inhibition of cyclooxygenase activity. Collectively, these data suggest that NSAIDs suppress the expression and assembly of catalytic subunits to inhibit proteasome activity and to increase $p27^{Kip1}$ protein levels.

Addition of Purified 26S Proteasome Counteracts NS398-Induced Inhibition of $p27^{Kip1}$ Degradation. If NSAIDs indeed suppressed the expression and assembly of proteasome subunits, it is obvious that $p27^{Kip1}$ degradation will be attenuated in NSAID-treated cells, and the addition of 26S proteasomes into the lysates of NSAID-treated cells should effectively promote $p27^{Kip1}$ degradation. Lysates prepared from vehicle- or NS398-treated cells were incubated with degradation buffer containing ubiquitin, ATP-generating system, and His6-tagged p27 ^{Kip1}. As shown in Fig. 7A., our data showed that degrading activity toward His6-tagged p27 ^{Kip1} was suppressed in cells treated with NS398 because His6-tagged p27 ^{Kip1} was almost completely degraded in the reaction buffer incubated with lysates of vehicle-treated cells after 1 h of incubation. However, the reaction buffer incubated with lysates of NS398-treated cells remained 30 to 50%

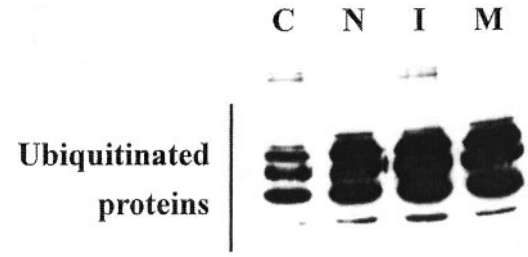

Fig. 2. NSAIDs induce the accumulation of ubiquitinated proteins. Cells were incubated with vehicle (C), 100 μM NS398 (N), 100 μM indomethacin (I), or 10 μM MG132 (M) for 24 h and then harvested for Western blot analysis and probing with antiubiquitin antibody.

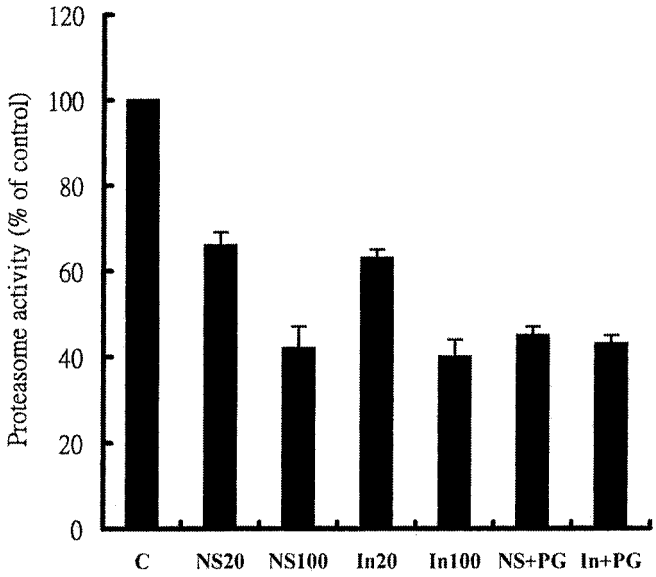

Fig. 3. NSAIDs inhibit proteasome activity in A549 cells. Cells were treated with 20 μM or 100 μM NS398 (NS20 and NS100, respectively) or indomethacin (In20 and In100, respectively) for 24 h and harvested for analysis of proteasome activity as described under *Materials and Methods*. In some experiments, 1 μM prostaglandin E_2 (PG) was coincubated with NS398 or indomethacin.

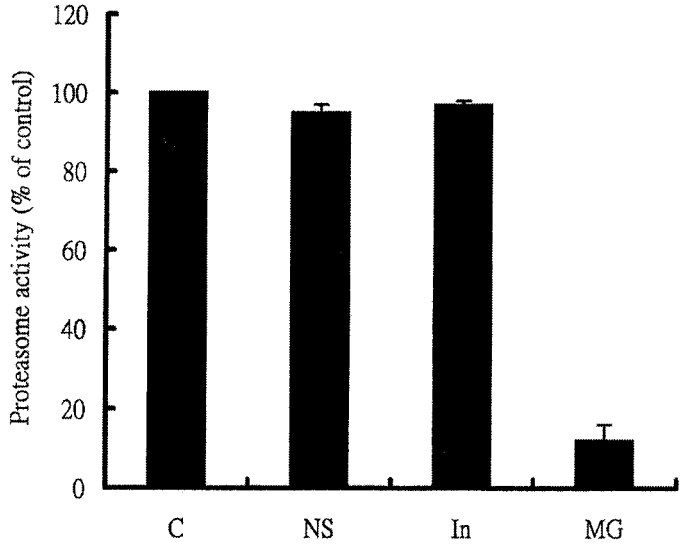

Fig. 4. NSAIDs are not direct enzymatic inhibitors for proteasomes. Purified 20S proteasome (0.5 μg) was diluted to 100 μl in a reaction buffer (50 mM Tris, pH 7.4, 1 mM dithiothreitol, 5 mM $MgCl_2$, and 2 mM ATP). To the reaction buffer was added 100 μM NS398 (N), 100 μM indomethacin (I), or 10 μM MG132 (M). The fluorogenic substrate Suc-LLVY-AMC (100 μM) was then added to samples and incubated at 37°C for 45 min. The reaction was terminated, and the intensity of fluorescence was measured by a fluorescent spectrophotometer.

of the added His6-tagged p27^{Kip1} after reaction as detected by immunoblotting. Figure 7B showed that minor p27^{Kip1} degrading activity was detected in NS398-treated cell lysates, and His6-tagged p27^{Kip1} protein was degraded gradually after incubation. However, the addition of purified 26S proteasome obviously promoted degradation, and His6-tagged p27^{Kip1} was almost undetectable after incubation for 15 min.

Discussion

Our previous work demonstrated that NS398 up-regulates p27^{Kip1} levels by inhibiting protein degradation (Hung et al., 2000). Two recent studies also indicated that another NSAID, aspirin, might up-regulate p27^{Kip1} in vascular smooth muscle cells and colon cancer cells (Marra et al., 2000; Kralj et al., 2001). In this study, we sought to elucidate

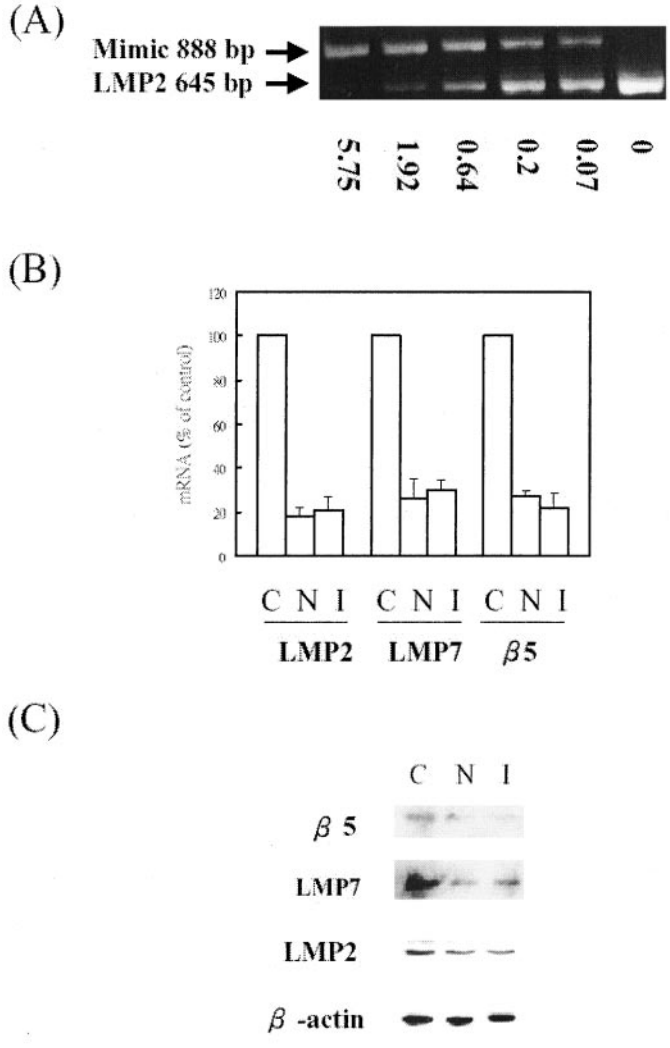

Fig. 5. NSAIDs suppress the expression of chymotrypsin-like catalytic subunits of proteasome. A, an RT-competitive PCR was performed as described under *Materials and Methods* to quantify the amount of LMP2 in A549 cells. B, cells were treated with vehicle (C), 100 μM NS398 (N), or 100 μM indomethacin (I) for 24 h. β5, LMP7, and LMP2 mRNA levels were investigated by RT-competitive PCR. C, the protein level of β5, LMP7, and LMP2 was analyzed by Western blot analysis, and β-actin was used as an internal control.

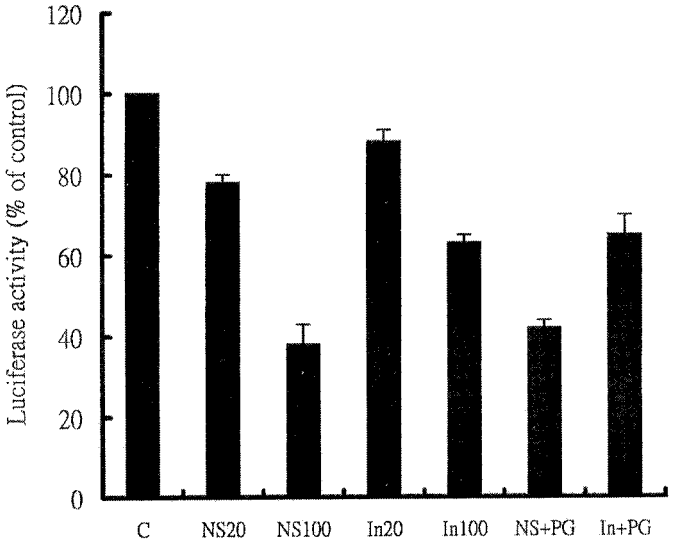

Fig. 6. NSAIDs directly suppress LMP2 promoter activity. Cells were cotransfected with 2 μg of LMP2 promoter-luciferase construct and 2 μg of pCMV–β-galactosidase plasmid by using the LipofectAMINE method. Cells were incubated in the absence or presence of 100 μM NS398 (N) or 100 μM indomethacin (In) for 24 h, and luciferase activity was examined. Luciferase activity was normalized with β-galactosidase activity and expressed as a percentage of the control. Results shown are from three independent experiments of duplicate samples.

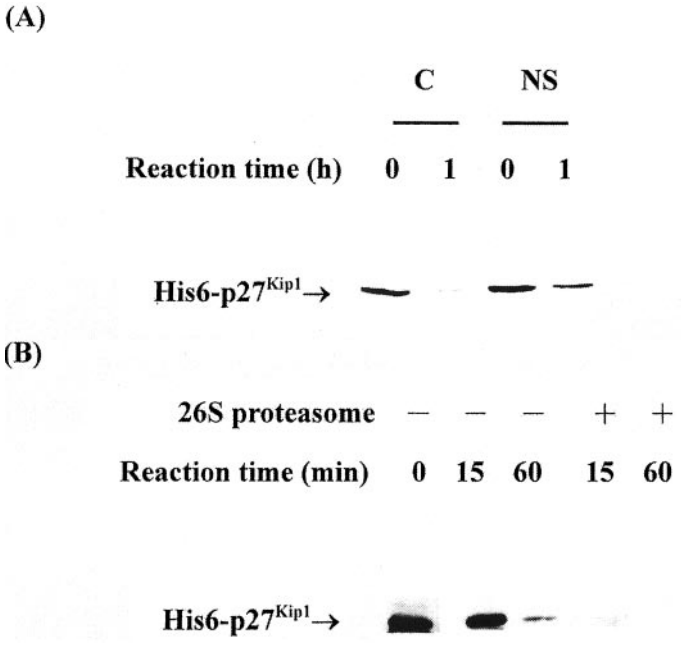

Fig. 7. The addition of 26S proteasome counteracts NS398-induced inhibition of p27^{Kip1} degradation. A, cells were treated with vehicle (C) or 100 μM NS398 (N) for 24 h. Cells were suspended in ice-cold double-distilled water and were frozen and thawed three times to lyse the cells. Cell lysates (10 μg) were diluted to 100 μl in a reaction buffer (50 mM Tris, pH 7.4, 1 mM dithiothreitol, 5 mM $MgCl_2$, and 2 mM ATP), and 0.5 μg of His6-tagged p27^{Kip1} was added. The reactions were carried out at 37°C for 1 h and terminated by adding SDS sample buffer. Each reaction mixture was subjected to SDS-PAGE and probed with anti-His6 antibody to monitor His6-tagged p27^{Kip1} protein levels in the mixture. C, lysates of NS398-treated cells were prepared and incubated with (+) or without (−) purified 26S proteasome, and in vitro degradation assays were performed as described above. Each reaction mixture was subjected to SDS-PAGE and probed with anti-His6 antibody to monitor the His6-tagged p27^{Kip1} protein levels in the mixture.

the molecular mechanism by which NSAIDs modulate $p27^{Kip1}$ degradation, and our results demonstrate that NSAIDs increase $p27^{Kip1}$ levels via the inhibition of proteasome activity.

Several novel findings are noteworthy. First, our results indicate that NSAIDs may up-regulate $p27^{Kip1}$ levels by inhibiting proteasome-mediated degradation to suppress tumor growth and to provide a molecular basis for NSAID-induced growth inhibition. Additionally, these results may be of clinical significance. Recent studies have demonstrated that more than 70% of non–small-cell lung cancer tumors showed reduced $p27^{Kip1}$ protein levels (Catzavelos et al., 1999; Hayashi et al., 2000). Moreover, the loss of this CDKI is linked with poor clinical outcome (Yatabe et al., 1998). It is rational to suggest that natural or synthetic agents which can effectively up-regulate $p27^{Kip1}$ expression will be considered to be useful drugs for the prevention or treatment of lung cancer. Our results suggest that NSAIDs fit this category and may be a novel class of chemopreventive drugs for lung cancer. Indeed, epidemiological investigations indicated that NSAID use is associated with reduced lung cancer incidence (Schreinemachers and Everson, 1994; Nelson, 1995).

Second, a number of works showed that proteasome inhibitors exert a potent anticancer effect in vitro and in vivo (Shinohara et al., 1996; Kitagawa et al., 1999; Fan et al., 2001). To date, most of the proteasome inhibitors are known to suppress proteasome activity by modifying the critical residue in the active sites or by competing with the substrate-binding sites in the catalytic subunits. Our study provides new evidence that proteasome activity can also be regulated by modulating the expression and assembly of proteasome subunits. A recent study reported another novel mechanism of proteasome inhibition (Zaiss et al., 1999). The authors demonstrated that the proteasome inhibitor PI31 may inhibit proteasome activity via competitive inhibition of the proteasome activator PA28. Thus, besides direct enzymatic inhibition, intracellular proteasome activity can also be affected by different mechanisms. An important and unresolved question is why the inhibition of proteasome activity by NSAIDs predominantly affects $p27^{Kip1}$ degradation. One possible explanation is that $p27^{Kip1}$ is very sensitive to the chymotrypsin-like activity of proteasome. Because NSAIDs preferentially suppress the expression of $\beta5$, LMP7, and LMP2, it is possible that the accumulation of $p27^{Kip1}$ will be detected easily and quickly after NSAID treatment when the proteolysis of other cell-cycle regulatory proteins is still unaltered. Indeed, a recent study showed that a dietary polyphenol, tannic acid, specifically inhibited the chymotrypsin-like activity of proteasomes and predominantly increased $p27^{Kip1}$ protein levels in tumor cells (Nam et al., 2001). In addition, the rapid accumulation of $p27^{Kip1}$ has also been observed in cells incubated with various dipeptidyl proteasome inhibitors (An et al., 1998; Sun et al., 2001).

Third, this study indicates that NS398 and indomethacin, in addition to being anti-inflammatory drugs, are also potent proteasome inhibitors. It is possible that proteasome inhibitors may exhibit an anti-inflammatory effect, per se. Indeed, a recent work demonstrated that the proteasome inhibitor epoxomicin is a potent anti-inflammatory agent in vivo (Meng et al., 1999). Therefore, the molecular targets and clinical usefulness of NSAIDs and proteasome inhibitors are more broad and complex than that was originally believed.

The mechanism by which NSAIDs regulate $\beta5$, LMP7, and LMP2 expression is not presently clear. A number of transcription-factor binding sites, including interferon-γ regulatory factor, simian virus 40 promoter factor 1, nuclear factor-κB, and activator protein-1, have been identified in the human LMP2 promoter (Wright et al., 1995), and elucidating the critical elements and signaling pathways that mediated the effect of NSAIDs on the expression of these catalytic subunits will be an important topic for future study. We clarified the mechanism by which NSAIDs up-regulate $p27^{Kip1}$ protein levels in lung cancer cells, and our results may be helpful for the development of new strategies for the prevention of lung cancer.

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