2,6-Diisopropylphenol Protects Osteoblasts from Oxidative Stress-Induced Apoptosis through Suppression of Caspase-3 Activation

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ABSTRACT: **2,6-Diisopropylphenol is an intravenous anesthetic agent used for induction and maintenance of anesthesia. Since it is similar to α-tocopherol, 2,6-diisopropylphenol may have antioxidant effects. Osteoblasts play important roles in bone remodeling. In this study, we attempted to evaluate the protective effects of 2,6-diisopropylphenol on oxidative stress-induced osteoblast insults and their possible mechanisms, using neonatal rat calvarial osteoblasts as the experimental model. Clinically relevant concentrations of 2,6-diisopropylphenol (3 and 30 μM) had no effect on osteoblast viability. However, 2,6-diisopropylphenol at 300 μM time-dependently caused osteoblast death. Exposure to sodium nitroprusside (SNP), a nitric oxide donor, increased amounts of nitrite in osteoblasts. 2,6-Diisopropylphenol did not scavenge basal or SNP-releasing nitric oxide. Hydrogen peroxide (HP) enhanced levels of intracellular reactive oxygen species in osteoblasts. 2,6-Diisopropylphenol significantly reduced HP-induced oxidative stress. Exposure of osteoblasts to SNP and HP decreased cell viability time-dependently. 2,6-Diisopropylphenol protected osteoblasts from SNP- and HP-induced cell damage. Analysis by a flow cytometric method revealed that SNP and HP induced osteoblast apoptosis. 2,6-Diisopropylphenol significantly blocked SNP- and HP-induced osteoblast apoptosis. Administration of SNP and HP increased caspase-3 activities. However, 2,6-diisopropylphenol significantly decreased SNP- and HP-enhanced caspase-3 activities. This study shows that a therapeutic concentration of 2,6-diisopropylphenol can protect osteoblasts from SNP- and HP-induced cell insults, possibly via suppression of caspase-3 activities.**

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Ann. N.Y. Acad. Sci. 1042: 448–459 (2005).
doi: 10.1196/annals.1338.038

KEYWORDS: **2,6-diisopropylphenol; osteoblasts; nitric oxide; hydrogen peroxide; apoptosis; caspase-3**

INTRODUCTION

2,6-Diisopropylphenol, an intravenous anesthetic agent, has the advantages of rapid onset, a short duration of action, and rapid elimination.[1] This intravenous anesthetic agent is widely used for induction and maintenance of anesthesia in surgical procedures. Structurally, 2,6-diisopropylphenol is similar to α-tocopherol with a phenol group so it has been implicated as having antioxidant roles.[2] Previous studies reported that 2,6-diisopropylphenol can protect thymocytes and erythrocytes from peroxinitrite- and 2,2′-azo-bis(2-amidinopropane) dihydrochloride–induced apoptosis.[3,4] Studies in human plasma and rat liver mitochondria revealed that 2,6-diisopropylphenol has antioxidant effects against lipid peroxidation.[2,5] Our previous study further demonstrated that 2,6-diisopropylphenol can protect macrophages from nitric oxide (NO)-induced damage.[6]

2,6-Diisopropylphenol is also used as an inducing or maintaining agent for patients undergoing orthopedic surgery.[7] Osteoblasts play critical roles in bone remodeling.[8] Reactive oxygen species (ROS) are one of the important factors that contribute to osteoblast metabolism.[8] NO and hydrogen peroxide (HP) are two typical ROS. In response to stimulation by inflammatory cytokines, osteoblasts synthesize massive amounts of NO and HP.[9,10] Elevation of NO and HP leads to cell injury.[11,12] Our previous study showed that NO can induce osteoblast apoptosis.[12] Caspase-3 is a proteolytic enzyme.[13] Activation of caspase-3 can drive cells to undergo apoptosis. In this study, we attempted to verify the protective effects of propofol on NO- and HP-induced osteoblast insults and their possible mechanisms.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

Osteoblasts were prepared from 3-day-old Wistar rat calvaria following an enzymatic digestion method described previously.[14] Osteoblasts were seeded in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin in tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2.

2,6-Diisopropylphenol was purchased from Aldrich (Milwaukee, WI). 2,6-Diisopropylphenol was stored under nitrogen, protected from light, and freshly prepared by dissolving it in dimethyl sulfoxide (DMSO) for each independent experiment. DMSO in the medium was kept to less than 0.1% to avoid the toxicity of this solvent to osteoblasts. Sodium nitroprusside (SNP) and HP were purchased from Sigma (St. Louis, MO). SNP and HP were freshly prepared by dissolving them in phosphate-buffered saline (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) for each independent experiment. Osteoblasts were exposed to various concentrations of 2,6-diisopropylphenol, SNP, and HP for different time intervals.

Assay of Cell Viability

Cell viability was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.[15] In brief, osteoblasts (2×10^4) were seeded in 96-well tissue culture plates overnight. After drug treatment, osteoblasts were cultured with new medium containing 0.5 mg/mL MTT for 3 more hours. The blue formazan products in osteoblasts were dissolved in DMSO and spectrophotometrically measured at a wavelength of 570 nm.

Assay of Nitrite Production

After drug treatment, amounts of nitrite, an oxidative product of NO, in the culture medium of osteoblasts were detected following a procedure described in a technical bulletin of Promega's Griess Reagent System (Promega, Madison, WI).

Quantification of Intracellular Reactive Oxygen Species

Intracellular levels of ROS were quantified following a method as described previously.[16] In brief, osteoblasts (5×10^5) were cultured in 12-well tissue culture plates overnight. 2′,7′-Dichlorofluorescin diacetate, an ROS-sensitive dye, was co-treated with 2,6-diisopropylphenol or HP for different time intervals. Osteoblasts were harvested and suspended in phosphate-buffered saline. Fluorescence intensities of osteoblasts were quantified using a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA).

Analysis of Apoptotic Cells

Apoptotic osteoblasts were determined using propidium iodide to detect DNA fragments in nuclei according to a method as described previously.[17] After drug administration, osteoblasts were harvested and fixed in cold 80% ethanol. Following centrifugation and washing, fixed cells were stained with propidium iodide and analyzed by a FACScan flow-cytometer (Becton Dickinson) on the basis of 560-nm dichroic mirror and 600-nm band-pass filter.

Assay of Caspase-3 Activity

Activity of caspase-3 was determined by a fluorogenic substrate assay. In brief, cell extracts were prepared by lysing osteoblasts in a buffer containing 1% Nonidet P-40, 200 mM NaCl, 20 mM Tris/HCl, pH 7.4, 10 mg/mL leupeptin, 0.27 U/mL aprotinin, and 100 mM PMSF. Caspase-3 activity is determined by incubating cell lysates (25 mg total protein) with 50 mM fluorogenic substrate in a 200 mL cell-free system buffer comprising 10 mM Hepes, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF, and 1 mM dithiothreitol. Intensities of fluorescent products in cells were measured by a spectrofluorometer.

Statistical Analysis

Statistical differences between the drug-treated groups were considered significant when the *P* value of Duncan's multiple-range test was less than 0.05. Statistical

TABLE 1. Concentration- and time-dependent effects of 2,6-diisopropylphenol (DIP) on osteoblast viability

	Cell viability (O.D. values at 570 nm)		
DIP (μM)	1 h	6 h	24 h
0	0.724 ± 0.083	0.768 ± 0.062	0.868 ± 0.072
3	0.760 ± 0.079	0.783 ± 0.081	0.842 ± 0.071
30	0.746 ± 0.086	0.760 ± 0.079	0.885 ± 0.063
300	0.717 ± 0.068	0.614 ± 0.063*	0.564 ± 0.065*

Each value represents the mean ± SEM for $n = 9$. *Values significantly differ from the respective control, $P < 0.05$.

TABLE 2. Effects of 2,6-diisopropylphenol (DIP) and sodium nitroprusside (SNP) on nitrite production

	Nitrite (μM)		
Treatment	1 h	6 h	24 h
Control	5.3 ± 1.1	4.2 ± 0.6	4.6 ± 0.6
DIP	4.7 ± 0.7	5.0 ± 0.9	3.9 ± 0.8
SNP	28.1 ± 5.6*	40.6 ± 7.1*	56.5 ± 8.2*
DIP + SNP	26.8 ± 6.4*	31.3 ± 6.9*	35.2 ± 6.6*

Each value represents the mean ± SEM for $n = 6$. *Values significantly differ from the respective control, $P < 0.05$.

analysis between the drug-treated groups over time was carried out by two-way ANOVA.

RESULTS

Treatment with 3 and 30 μM 2,6-diisopropylphenol for 1, 6, and 24 h was not cytotoxic to osteoblasts (TABLE 1). Osteoblast viability was not affected following exposure to 300 μM 2,6-diisopropylphenol for 1 h. However, when the administered time intervals reached 6 and 24 h, 2,6-diisopropylphenol significantly decreased osteoblast viability by 20% and 35%, respectively (TABLE 1).

Administration of 2 mM SNP in osteoblasts for 1, 6, and 24 h enhanced amounts of nitrite in the culture medium by 5.3-, 9.7-, and 12.3-fold, respectively (TABLE 2). Exposure to 30 μM 2,6-diisopropylphenol for 1, 6, and 24 h did not affect nitrite production. Co-treatment of osteoblasts with 2,6-diisopropylphenol and SNP did not influence the amounts of nitrite in the culture medium.

Treatment of osteoblasts with 25 μM HP for 1, 6, and 24 h significantly increased intracellular levels of ROS by 4.5-, 4.4-, and 3.4-fold, respectively (TABLE 3). Administration of 2,6-diisopropylphenol did not affect intracellular ROS levels. Co-treatment with 2,6-diisopropylphenol and HP for 1 and 6 h significantly reduced HP-

TABLE 3. Effects of 2,6-diisopropylphenol (DIP) and hydrogen peroxide (HP) on levels of intracellular reactive oxygen species (ROS)

Treatment	ROS, fluorescence intensities		
	1 h	6 h	24 h
Control	16 ± 3	19 ± 5	15 ± 2
DIP	13 ± 2	4 ± 3	2 ± 1
HP	72 ± 16*	82 ± 17*	51 ± 10*
DIP + HP	37 ± 11*†	51 ± 9*†	43 ± 10*

Each value represents the mean ± SEM for $n = 6$. *Values significantly differ from the respective control, $P < 0.05$. †Values significantly differ from the HP-treated groups, $P < 0.05$.

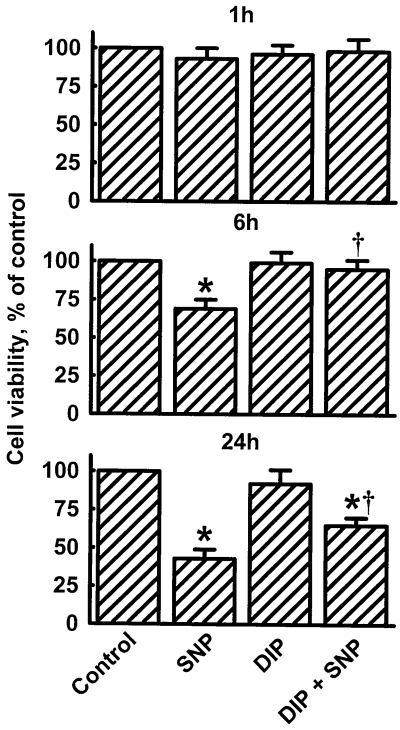

FIGURE 1. Protective effects of 2,6-diisopropylphenol (DIP) on sodium nitroprusside (SNP)-induced osteoblast death. Osteoblasts prepared from neonatal rat calvaria were exposed to 30 μM DIP, 2 mM SNP, and a combination of DIP and SNP. Cell viability was analyzed by the MTT assay. Each value represents the mean ± SEM for $n = 12$. *Values significantly differ from the respective control, $P < 0.05$. †Values significantly differ from the SNP-treated groups, $P < 0.05$.

induced intracellular ROS production by 49% and 38%, respectively. Administration of 2,6-diisopropylphenol and HP for 24 h had no effect on HP-induced intracellular ROS production (TABLE 3).

Administration of osteoblasts with SNP for 1 h was not cytotoxic to osteoblasts (FIG. 1). Viability of osteoblasts was respectively reduced by 31% and 57% following administration of SNP for 6 and 24 h. Exposure to 2,6-diisopropylphenol for 1, 6, and 24 h did not affect cell viability. Co-treatment with 2,6-diisopropylphenol and SNP for 6 h completely ameliorated SNP-induced cell death. In 24-h-treated osteo-

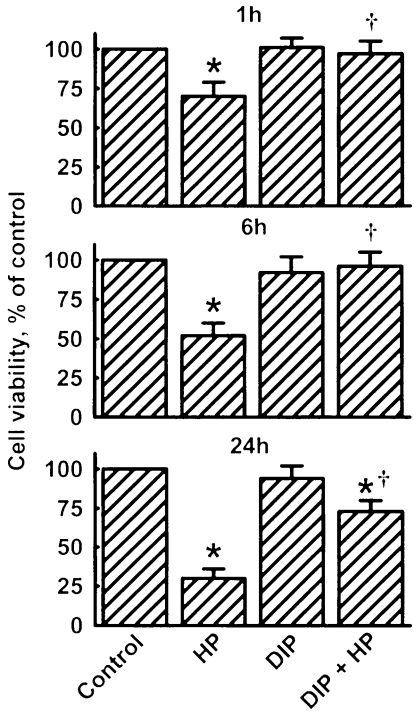

FIGURE 2. Protective effects of 2,6-diisopropylphenol (DIP) on hydrogen peroxide (HP)-induced osteoblast death. Osteoblasts prepared from neonatal rat calvaria were exposed to 30 μM DIP, 25 μM HP, and a combination of DIP and HP. Cell viability was analyzed by the MTT assay. Each value represents the mean ± SEM for $n = 12$. *Values significantly differ from the respective control, $P < 0.05$. †Values significantly differ from the SNP-treated groups, $P < 0.05$.

blasts, 2,6-diisopropylphenol caused a partial 22% reduction in SNP-induced cell death (FIG. 1).

Exposure of osteoblasts to HP for 1, 6, and 24 h significantly decreased osteoblast viability by 30%, 52%, and 70%, respectively (FIG. 2). 2,6-Diisopropylphenol did not affect cell viability. Co-treatment with 2,6-diisopropylphenol and HP for 1 and 6 h completely ameliorated HP-induced osteoblast death. Administration of 2,6-diisopropylphenol led to a 43% decrease in HP-induced cell death (FIG. 2).

Analysis by a flow cytometric method revealed that administration of SNP to osteoblasts for 1 h did not induce cell apoptosis (FIG. 3). When the administered time intervals reached 6 and 24 h, SNP significantly induced osteoblast apoptosis by 25% and 40%, respectively. Exposure to 2,6-diisopropylphenol had no effect on osteoblast apoptosis. Co-treatment with 2,6-diisopropylphenol and SNP for 6 and 24 h significantly blocked SNP-induced osteoblast apoptosis by 40% and 35%, respectively (FIG. 3).

Administration of HP to osteoblasts for 1, 6, and 24 h significantly induced osteoblast apoptosis by 15%, 35%, and 56%, respectively (FIG. 4). 2,6-Diisopropylphenol did not influence cell apoptosis. Co-treatment with 2,6-diisopropylphenol and HP, respectively, decreased HP-induced osteoblast apoptosis by 11%, 31%, and 25% (FIG. 4).

In untreated osteoblasts, low levels of caspase-3 activities were detected (FIG. 5). Administration of 2,6-diisopropylphenol had no effect on caspase-3 activity. Expo-

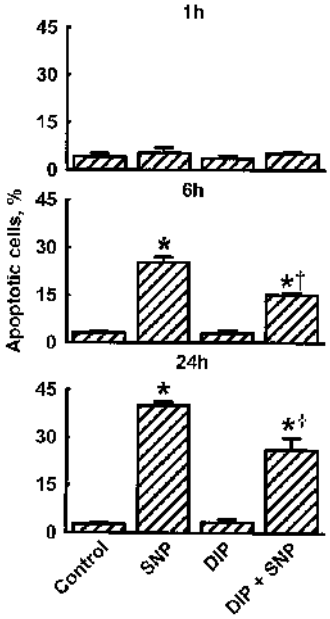

FIGURE 3. Protective effects of 2,6-diisopropylphenol (DIP) on sodium nitroprusside (SNP)-induced osteoblast apoptosis. Osteoblasts prepared from neonatal rat calvaria were exposed to 30 μM DIP, 2 mM SNP, and a combination of DIP and SNP. Apoptotic cells were determined using flow cytometry. Each value represents the mean ± SEM for $n = 6$. *Values significantly differ from the respective control, $P < 0.05$. †Values significantly differ from the SNP-treated groups, $P < 0.05$.

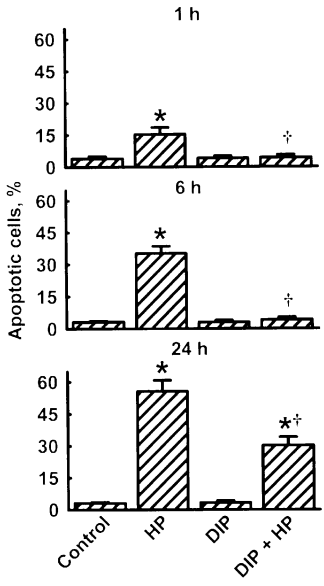

FIGURE 4. Protective effects of 2,6-diisopropylphenol (DIP) on hydrogen peroxide (H_2O_2)-induced osteoblast apoptosis. Osteoblasts prepared from neonatal rat calvaria were exposed to 30 μM DIP, 25 μM HP, and a combination of DIP and HP. Apoptotic cells were determined using flow cytometry. Each value represents the mean ± SEM for $n = 6$. *Values significantly differ from the respective control, $P < 0.05$. †Values significantly differ from the SNP-treated groups, $P < 0.05$.

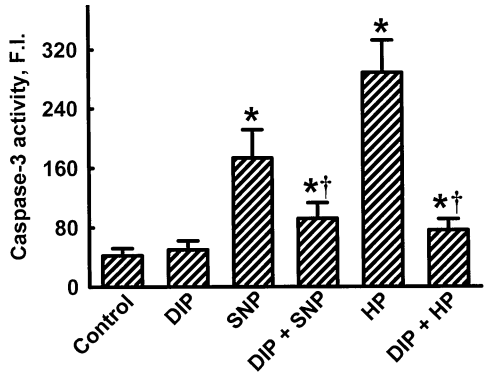

FIGURE 5. Effects of 2,6-diisopropylphenol (DIP) on sodium nitroprusside (SNP)- and hydrogen peroxide (HP)-induced caspase-3 activities. Osteoblasts prepared from neonatal rat calvaria were exposed to 30 μM DIP, 2 mM SNP, 25 μM HP, and a combination of DIP and SNP or DIP and HP. Caspase-3 activity was determined using a fluorogenic substrate assay. Each value represents the mean ± SEM for $n = 6$. *Values significantly differ from the respective control, $P < 0.05$. †Values significantly differ from the SNP- or HP-treated groups, $P < 0.05$. F.I., fluorescence intensities.

sure to SNP and HP significantly increased caspase-3 activity by 131% and 246%, respectively. 2,6-Diisopropylphenol caused a significant 47% decrease in SNP-enhanced caspase-3 activities. The HP-induced caspase-3 activity was significantly reduced by 74% following co-treatment with 2,6-diisopropylphenol and HP (FIG. 5).

DISCUSSION

2,6-Diisopropylphenol is one of the common intravenous anesthetic agents used for induction and maintenance of anesthesia during surgical procedures, including orthopedic operations.[1,7] Osteoblasts are involved in bone remodeling.[8] This study showed that administration of 3 and 30 μM 2,6-diisopropylphenol was not cytotoxic to osteoblasts. However, viability of osteoblasts was decreased time-dependently following administration of 300 μM 2,6-diisopropylphenol. Our previous study presented similar results of 2,6-diisopropylphenol at therapeutic concentrations having no effect on macrophage viability.[16] However, 2,6-diisopropylphenol at a high concentration (300 μM) increased lactate dehydrogenase release and led to an arrest of the cell cycle in the G1/S phase. 2,6-Diisopropylphenol at 30 μM is within the range of clinical plasma concentrations.[18] Therefore, clinically relevant concentrations of 2,6-diisopropylphenol are not harmful to osteoblasts and macrophages.

SNP significantly increased nitrite production in osteoblasts. SNP can be decomposed to NO under light exposure or in the presence of a biological reducing system.[12,19] The use of SNP in this study had a biochemical advantage because it provides NO for an investigation of signaling transduction pathways without interfering with NO synthase-involved second messenger systems. Nitrite is an oxidative

product of NO. Increases in amounts of nitrite correspond to enhanced levels of NO in cells. Thus, administration of SNP to osteoblasts significantly increases levels of intracellular NO. Osteoblasts can constitutively produce NO.[20] Low levels of nitrite were detectable in untreated osteoblasts. 2,6-Diisopropylphenol did not decrease basal or SNP-released NO levels. Our previous study demonstrated that 2,6-diisopropylphenol downregulates NO biosynthesis via inhibition of inducible NO synthase in lipopolysaccharide-activated macrophages.[21] Therefore, 2,6-diisopropylphenol cannot directly scavenge exogenous NO, but reduces endogenous NO production through inhibition of the NO-synthesizing enzyme.

Administration of HP to osteoblasts increased intracellular levels of ROS. This study used DCFH-DA dye to quantify ROS. The major forms of ROS reacting with this dye are peroxides.[22] HP is one of the cellular peroxides. Thus, HP itself can enhance intracellular ROS levels in osteoblasts. A previous study reported that HP leads to depolarization of the mitochondrial membrane potential and promotes apoptotic factor release from the mitochondria to the cytoplasm.[23] ROS are typical mitochondrial apoptotic factors.[24] The other possible reason that explains the enhancement of intracellular ROS in osteoblasts following HP administration may be the release of mitochondrial ROS via depolarization of the mitochondrial membranes. 2,6-Diisopropylphenol significantly decreased HP-enhanced intracellular ROS levels. 2,6-Diisopropylphenol has a phenol group that can scavenge ROS.[2] Previous studies revealed that 2,6-diisopropylphenol can directly scavenge lipid peroxyryl radicals, hydroxyl radicals, and superoxide.[2,25] The present study further shows that 2,6-diisopropylphenol can also directly scavenge HP.

SNP decreased osteoblast viability time-dependently. NO can be decomposed from SNP.[19] An elevation of NO levels increases cellular oxidative stress and results in cell damage.[9,12] Apoptotic analysis revealed that SNP induced osteoblast apoptosis time-dependently. NO is a critical bioregulator which induces apoptosis in different types of cells.[26] Thus, the SNP-induced osteoblast death mainly occurs via an apoptotic mechanism. This study shows that a therapeutic concentration of 2,6-diisopropylphenol (30 μM) can protect osteoblasts from SNP-induced cell insults and apoptosis. Because 2,6-diisopropylphenol did not decrease basal or SNP-released NO, this intravenous anesthetic agent protects osteoblasts against apoptosis through means other than the direct scavenging of NO. Following SNP administration, caspase-3 activity was significantly increased. Activation of caspase-3 causes proteolytic cleavage of key proteins and induces cell apoptosis.[13] Thus, the apoptosis induced in osteoblasts by SNP involves activation of caspase-3 activity. Co-treatment with 2,6-diisopropylphenol and SNP significantly reduced SNP-enhanced caspase-3 activity. In parallel with the suppression of caspase-3 activity, 2,6-diisopropylphenol significantly reduced SNP-induced osteoblast apoptosis. Therefore, 2,6-diisopropylphenol can block SNP-induced osteoblast apoptosis via suppression of caspase-3 activity.

HP decreased osteoblast viability time-dependently. HP is an ROS. HO enhances oxidative stress and leads to cell death.[11] In parallel with cell damage, HP induced osteoblast apoptosis in a time-dependent manner. Thus, HP induces osteoblast injury through an apoptotic pathway. Co-treatment of 2,6-diisopropylphenol and HP significantly decreased HP-induced osteoblast insults and apoptosis. 2,6-Diisopropylphenol can reduce HP-enhanced intracellular ROS. ROS are apoptotic factors involved in the regulation of cell death.[24] Thus, 2,6-diisopropylphenol can directly scavenge intra-

cellular ROS and protect osteoblasts from HP-induced insults. Administration of HP significantly increased osteoblast caspase-3 activity. Thus, HP induces osteoblast apoptosis mainly through activation of caspase-3 activity. 2,6-Diisopropylphenol significantly decreased HP-enhanced caspase-3 activity. Therefore, the other mechanism to explain the protective effects of 2,6-diisopropylphenol against HP-induced osteoblast apoptosis may occur via a reduction in caspase-3 activity.

The protective effects of 2,6-diisopropylphenol on osteoblasts from SNP- and HP-induced cell damage decreased with time. This time-dependent recovery was also observed in the 2,6-diisopropylphenol–involved reduction of intracellular ROS levels. 2,6-Diisopropylphenol can be progressively decomposed in aerobic conditions or after exposure to visible light.[1] This characteristic might explain why the 2,6-diisopropylphenol–induced protection of osteoblasts from SNP- and HP-induced injuries decreased with time. Another possible reason which explains the time-dependent decrease in the 2,6-diisopropylphenol–caused osteoblast protection is the metabolism of this anesthetic agent by cytochrome P450-dependent monooxygenase or UDP glucuronosyltransfease.[27] These two enzymes are detectable in osteoblasts.[28,29] Therefore, the lowered contents of 2,6-diisopropylphenol in osteoblasts, due to its metabolism by these related enzymes, may explain the decrease in the protective effects of this intravenous anesthetic agent on SNP- and HP-induced osteoblast insults.

CONCLUSION

This study shows that clinically relevant concentrations of 2,6-diisopropylphenol are not cytotoxic to osteoblasts. Administration of SNP and HP increases cellular oxidative stress and leads to osteoblast death via an apoptotic mechanism. 2,6-Diisopropylphenol at a therapeutic concentration can protect osteoblasts from SNP- and HP-induced cell insults and apoptosis. 2,6-Diisopropylphenol can directly scavenge HP-enhanced intracellular ROS without affecting SNP-releasing NO. Both SNP and HP activate capase-3 activities. 2,6-Diisopropylphenol can significantly reduce SNP- and HP-enhanced caspase-3 activities. Therefore, 2,6-diisopropylphenol can block HP-induced cell apoptosis through directly scavenging intracellular ROS and suppressing caspase-3 activity. However, a decrease in caspase-3 activity may be the major mechanism contributing to the protection of osteoblasts from SNP-induced cell apoptosis by 2,6-diisopropylphenol.

ACKNOWLEDGMENTS

This work was supported by the National Science Council (NSC93-2745-B-038-002-URD) and the Shin Kong Wu Ho-Su Memorial Hospital (SKH-TMU-92-32), Taipei, Taiwan.

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