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JNK/SAPK Is Required in Nitric Oxide-Induced Apoptosis in Osteoblasts

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Nitric oxide(NO) induces apoptosis in human osteoblasts. Treatment with exogenous NO donors, SNAP (*S*-Nitroso-*N*-acetylpenicillamine) and SNP (sodium nitroprusside), to MG-63 osteoblasts resulted in apoptotic morphological changes, as shown by a bright blue-fluorescent condensed nuclei and chromatin fragmentation by fluorescence microscope of Hoechst 33258-staining. The activities of caspase-9 and the subsequent caspase-3-like cysteine proteases were increased during NO-induced cell death. Pretreatment with Z-VAD-FMK (a pan-caspase inhibitor) or Ac-DEVD-CHO (a specific caspase-3 inhibitor) abrogated the NO-induced cell death. The NO donor markedly activated JNK, a stress-activated protein kinase in the human osteoblasts. This study showed that the inhibition of the JNK pathway markedly reduced NO-induced cell death. But neither PD98059 (MEK inhibitor) nor SB203580 (p38 MAPK inhibitor) had any effect on NO-induced death. Taken together, these results suggest that JNK/SAPK may be related to NO-induced apoptosis in MG-63 human osteoblasts.

Key words: Nitric oxide, *S*-Nitroso-*N*-acetylpenicillamine, Sodium nitroprusside, Apoptosis, MG-63 human osteoblasts

INTRODUCTION

Apoptosis is an active, energy-dependent mode of cell death of typical morphologic changes, such as nucleoplasmic and cytoplasmic condensation, the translocation of phosphatidylserine to the cell surface, the formation of extensive membrane blebs and novel membranous structures known as apoptotic bodies (Arends & Wyllie, 1991). Although the events underlying the initiation of apoptosis are not well understood, the processes appear to be closely associated with activation of one or more species of Ca^{++}/Mg^{++}-dependent endonucleases. They introduce double-stranded breaks at internucleosomal regions of mature DNA, resulting in the appearance of "laddered" electrophoretic patterns of oligonucleosomal DNA fragments on conventional agarose gels (Wyllie, 1980). Apoptosis thus appears to represent a specific and highly regulated program mediating self-destruction at the cellular level.

Nitric oxide (NO) is a labile free radical that is physiologically produced through the L-arginine/NO synthase (NOS) pathway (Moncada *et al.*, 1989). Of the three known isoforms of the NOS enzyme, two are low-output, Ca^{++}-dependent, constitutively expressed (nNOS/neuronal, eNOS/endothelial), while the third is inducible (iNOS), high-output, and Ca^{++}-independent (Forstermann *et al.*, 1991). NO is a highly reactive molecule that interacts with multiple cellular target moieties including guanylyl cyclase, cyclooxygenase, ribonucleotide reductase, membrane phospholipids, and DNA (Nathan, 1992). Cytokine-induced NO synthesis causes sustained vasodilation in septic shock (Kibourn *et al.*, 1990; Liew *et al.*, 1991), inhibits cell proliferation (Nunokawa & Tanaka, 1992), and mediates cytotoxicity (Bergmann *et al.*, 1992). In this study, exogenous NO induced programmed cell death in human osteoblasts-MG-63.

Apoptosis is caused by proteases known as "caspasesfor" cysteine aspartyl-specific proteases. Caspases constitute a family of intracellular cysteine proteases, which collaborate in proteolytic cascades, where caspases activate them-

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selves and each other (Thornberry *et al.*, 1997; Karanewsky *et al.*, 1998). Mitochondria also play important roles in apoptosis, releasing cytochrome-c (Cyt-c) into the cytosol, which then causes assembly of a multiprotein caspase-activating complex, referred to as the "apoptosome". The central component of the apoptosome is Apaf1, a caspase activating protein that oligomerizes upon binding Cyt-c and which specifically binds pro-caspase-9. The mitochondrial pathway for apoptosis is activated by myriad stimuli, including growth factor deprivation, reactive oxygen species including NO, Ca^{2+}over-load, DNA-damaging agents, and others (Reed, 2000; Reed and Paternostro, 1999; Deveraux and Reed, 1999; Du *et al.*, 2000).

Studies of signaling mechanisms have revealed that the mitogen-activated protein kinases (MAPKs) may play important roles in the pathways that regulate both growth and apoptosis. For example, the MAPK, extracellular signal regulated kinase (ERK), has been implicated as a growth promoter, displaying an apoptosis-protective effect in some cell types, while *N*-terminal c-Jun kinase (JNK) and/or p38 kinases have been observed to foster apoptosis in certain cells (Liu *et al.*, 2003; Paik *et al.*, 2003). However, it is not known whether MAPKs are involved in NO-induced apoptosis in osteoblasts. The aim of the present study was to determine whether signaling via MAPK pathways are involved in NO-induced apoptosis in osteoblasts.

MATERIALS AND METHODS

Materials

S-Nitroso-*N*-acetylpenicillamine (SNAP), 2-phenyl-4, 4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) and Hoechst 33258 were purchased from Sigma Chemical Co. (St. Louis, MO). JNK1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). *N*-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC), *N*-acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumain (Ac-YVAD-AMC) and Pansorbin were obtained from Calbiochem-Behring Corp (La Jolla, CA). Z-Val-Ala-Asp-7-amino-4-methylcoumain (Z-VAD-AMC) was purchased from Kamiya Bio Company (Seattle, WA).

Cell culture

The human osteoblasts, MG-63, were maintained in DMEM medium supplemented with 10% heat inactivated FBS, penicillin G (100 IU/mL), streptomycin (100 µg/mL), and L-glutamate (2 mM) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Exponentially growing MG-63 cells were seeded at 1×10^6 per 6-well plate or at 1×10^7 per 100-mm dish, after which the cells were exposed to the various agents indicated in the results section.

Morphological detection of apoptosis

Morphological evaluation of apoptotic cell death was performed as previously described with some modification (Chae *et al.*, 2000). Cells were fixed for 5 min in 3% paraformaldehyde in phosphate-buffered saline. After air-drying, cells were stained for 10 min in Hoechst 33258 (10 mg/mL), mounted in 50% glycerol containing 20 mM citric acid and 50 mM orthophosphate, and stored at -20°C before analysis. Nuclear morphology was evaluated using a Zeiss IM 35 fluorescent microscope.

Measurement of caspase-3paraformaldehyde and 9 like protease activities

MG-63 osteoblasts were lysed in a lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 1 mM PMSF, 1 µM aprotinin, 1 µM leupeptin, 2 mM dithiothreitol, 10 mM Tris/HCl, pH 8.0) on ice for 30 min and cleared at 15,000×g for 15 min. The activities of caspase-1 and -3 like proteases were measured by proteolytic cleavages of substrates including 100 µM of acetyl-DEVD-AMC and acetyl-LEHD-AFC, respectively. These fluorogenic substrates and AMC as a control were solubilized in the assay buffer containing 100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 µM aprotinin, 1 µM leupeptin, and 2 mM dithiothreitol. After incubation at 37°C for 30 min in the dark, fluorescence from the lysates was measured with a fluorometer (Molecular Devices, Palo Alto, CA) at dual wavelength of 380 nm of excitation wavelength and 460 nm of emission wavelength.

In vitro immunocomplex kinase assay for the activities of JNK/SAPK

The effect of NO on JNK/SAPK was assessed using MG-63 cell cultures (5×10^6 cells/100-mm dish). For JNK assays, cultures were extracted in a buffer containing 10 mM sodium o-vanadate and 20 µg/mL aprotinin. After brief centrifugation, extracts were incubated for 2 h at 4°C with anti-JNK antibody (Santa Cruz Biotechnology), and immune complex kinase assay was carried out using the appropriate substrates. Briefly, reactions were initiated by the addition of 1 µg of GST-c-jun for JNK and 6 µM [γ-^{32}P] ATP (5000 Ci/mmol) in a final volume of 30 µL of kinase buffer (20 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 2 mM dithiothreitol, 20 µM ATP). After 30 min at 25°C, the reactions were terminated by the addition of Laemmli sample buffer, and the phosphorylation level of substrate proteins was evaluated by SDS-polyacrylamide gel electrophoresis followed by autoradiography and phospho-image analyses. In each experiment, three identically treated cultures (1.5×10^6 cells/35-mm dish) were used for each treatment.

Plasmid construction and transfections

For expression of JNK1, full-length wild type JNK1 [26] was amplified by PCR from the human brain cDNA library and inserted in-frame into the pcDNA3 vector (Invitrogen, San Diego, CA). To prepare the kinase inactive form of JNK1 (JNK (KD)), the replacement of Lys-55 with Arg in wild type JNK1 was performed by two-step PCR mutagenesis and inserted into the pcDNA3 vector. The nucleotide sequence of all PCR-generated inserts was verified by using an automated sequencing system (Applied Biosystems Inc. Foster City, CA). After transfection into MG-63 cells with vector plasmids, the transfected cells were cultured in selective medium containing 600 μg/mL of G-418 for 4 weeks. Ectopic expression of JNK1 was confirmed by western blotting with anti-JNK antibody (Santa Cruz Biotechnology).

Statistical analysis

Statistical analyses were performed using paired Students *t*-test. Differences were considered significant at $P<0.05$.

RESULTS

JNK is a key factor on NO-induced apoptosis in MG-63 osteoblasts

To determine whether MAPKs signal transduction pathways are important in NO-induced apoptosis, we subcloned JNK1- (one of the MAPKs) DNA into the eukaryotic expression vector, pcDNA3, and generated JNK (KD), JNK (WT) and pcDNA3 transfectant MG-63 cells. These JNK (KD) transfectant cells showed markedly reduced NO donors (SNP and SNAP)-induced cell death (Fig. 1A). The cells were stained with Hoechst 33258 (blue fluorescence). Treatment with SNAP or SNP resulted in apoptotic morphological changes, as shown by a brightly blue-fluorescent condensed nuclei and chromatin fragmentation by fluorescence microscope (Fig. 1B). These JNK (KD) trans-

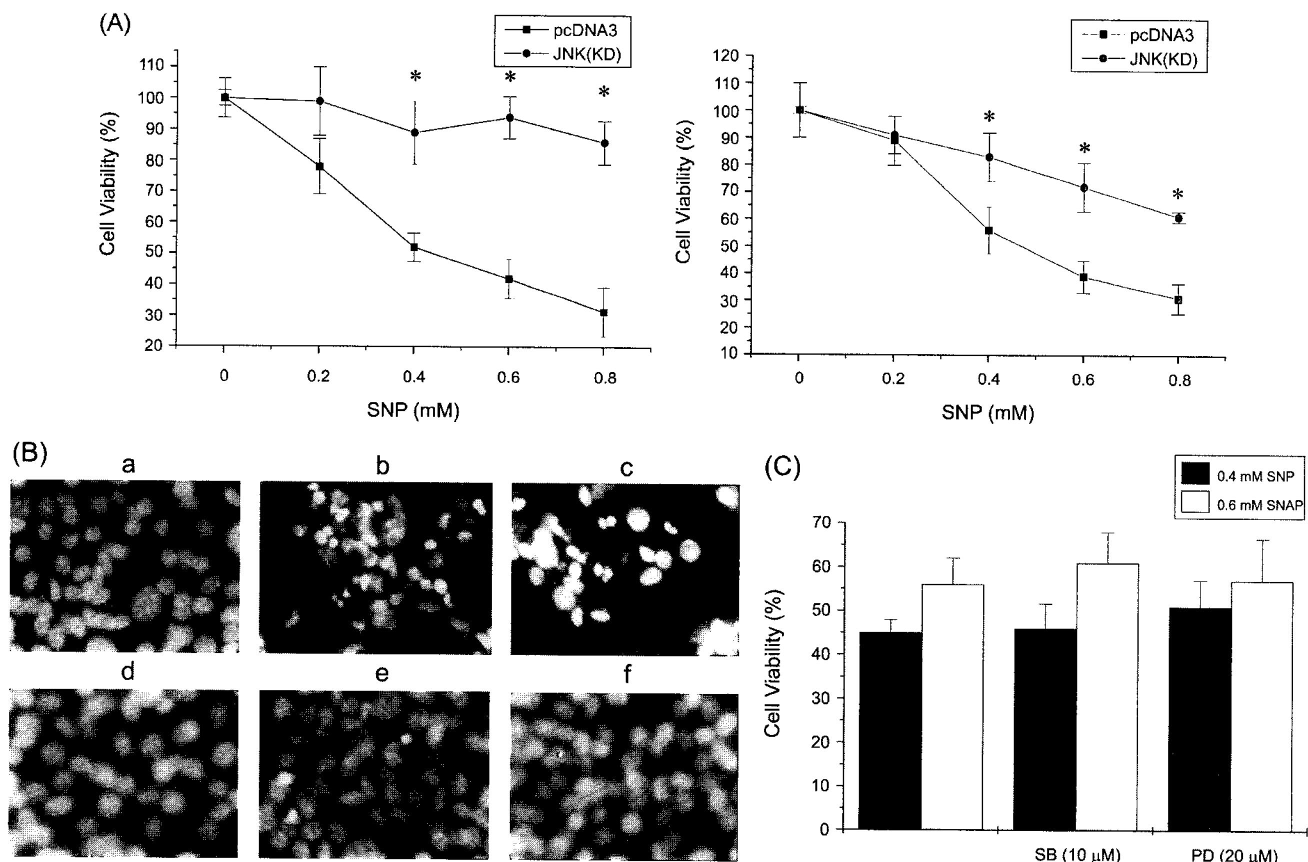

Fig. 1. Nitric oxide induces apoptosis in MG-63 osteoblasts. (A) Either pcDNA or JNK (KD)-transfectant cells were subjected to various concentrations of SNP (upper) or SNAP (lower) (0, 0.2, 0.4, 0.6 and 0.8 mM). Data are represented as mean±S.E. (n=4). $*P<0.05$ versus pcDNA 3 transfectants. (B) The cells were stained with Hoechst 33258 and observed with a fluorescence microscope. Nuclei were assessed in an Olympus OMT2 inverted fluorescence microscope, using a long working-distance objective, at a final magnification of 400. a: untreated pcDNA cells, b: SNAP (0.6 mM)-treated pcDNA cells, c: SNP (0.4 mM)-treated pcDNA cells, d: untreated JNK (KD) cells, e: SNAP (0.6 mM)-treated JNK (KD) cells, f: SNP (0.4 mM)-treated JNK (KD) cells. (C) Cells were pretreated with PD 98059 or SB203580 and then were exposed to 0.4 mM SNP or 0.6 mM SNAP. MTT assay was performed as described in Materials and Methods. Data are represented as mean ±S.E. (n=4).

fectant cells showed markedly reduced NO donors (SNP and SNAP)-induced apoptotic cell number. Then, we examined the effect of pretreatment of PD98059, an MEK inhibitor or SB203580, a p38 MAPK inhibitor, in the osteoblast cultures exposed to either 0.4 mM SNP or 0.6 mM SNAP. Neither of the two agents had any regulatory effect on the cell death (Fig. 1C). These results suggest that JNK plays a key role on NO-induced apoptosis in osteoblasts.

The inhibition of JNK regulates NO-induced caspase activation in osteoblasts

Caspases collaborate in proteolytic cascades, where caspases activate themselves and each other (Karanewsky *et al.*, 1998; Reed, 2000). Within these proteolytic cascades, caspases can be positioned as either upstream "initiators" or downstream "effectors" of apoptosis. Therefore, the activities of caspase-3 like protease (effector caspase) and caspase-9 like protease (initiator caspase) were measured. As shown in Fig. 2A and B, both caspase-3 and -9 like protease activities were increased in a time dependent manner and reached a maximum level at 16 h after the addition of SNP (0.4 mM) or SNAP (0.6 mM). But caspase-1 activity was not affected by either of these NO donors (data not shown). As expected, these JNK (KD) transfectant cells markedly reduced NO donors (SNP and SNAP)-induced activation of both caspase-3 and -9 (Fig. 2A and B). These results suggest that JNK plays a key role on NO-induced caspase-9 and subsequent caspase-3 activation in osteoblasts.

Fig. 2C shows that the pretreatment of osteoblasts with Z-VAD-FMK (100 μM) abrogated the NO-induced cell death. However, pretreatment with Ac-YVAD-CHO, a caspase-1 inhibitor, did not block the apoptosis (data not shown). It also showed that both SNAP and SNP-induced cell death were mediated by NO, because pretreatment with the NO scavenger, 2-phenyl-4,4,5,4,-tetramethylimidazoline-1-oxyl-3-oxide (PTIO, 30 mM), abrogated the cell damage.

Activations of JNK/SAPK in SNAP-treated MG-63 osteoblasts

Next, we examined JNK/SAPK activation for up to 4 h after the treatment of SNAP (0.6 mM). Kinase activity for JNK was measured by immunocomplex kinase assays with GST-c-Jun as a substrate. To determine whether the JNK/SAPK signal transduction pathway is important in NO-induced apoptosis, we used JNK (KD), JNK (WT) and pcDNA3 transfectant MG-63 cells. These JNK (KD) transfectant cells showed that GST-c-Jun phosphorylation was significantly inhibited in JNK(KD)-transfectant cells (Fig. 3). Therefore, these results suggest that the activation of JNK is involved in the NO-induced death of MG-63 cells. It has been reported that JNK kinase is a well-known stress-activated kinase, a major component of various stress-inflammatory cytokines, ischemia, ATP depletion,

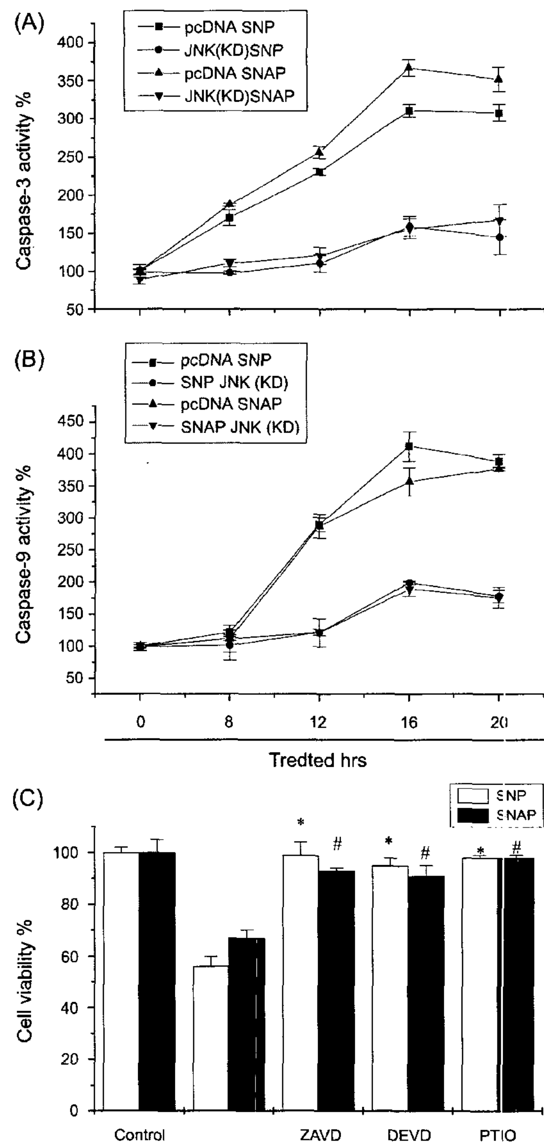

Fig. 2. Nitric oxide induces the activation of caspase-9 and subsequent-3 like proteases in osteoblasts. (A) Either pcDNA or JNK (KD)-transfectant cells were exposed to SNP (0.4 mM) or SNAP (0.6 mM) for various times. Then, the activities of caspase-3 proteases were measured by using acetyl-DEVD-AMC fluorogenic peptide as a substrate. (B) Either pcDNA or JNK (KD)-transfectant cells were exposed to SNP (0.4 mM) or SNAP (0.6 mM) for various times. Then, the activities of caspase-9 proteases were measured by using acetyl-LEHD-AMC fluorogenic tetrapeptide as a substrate. Results are expressed with the mean±S.E. of experiments conducted in quadruplicate. (C) Cells were incubated with 0.4 mM SNP or 0.6 mM of SNAP in the presence of Ac-DEVD-CHO (100 μM), Ac-ZVAD-FMK (100 mM) or PTIO (30 mM). Then, MTT assay was performed as described in Materials and Methods. Data are represented as mean±S.E. (n=4). **P*<0.05 versus SNP-treated. #*P*<0.05 versus SNAP-treated.

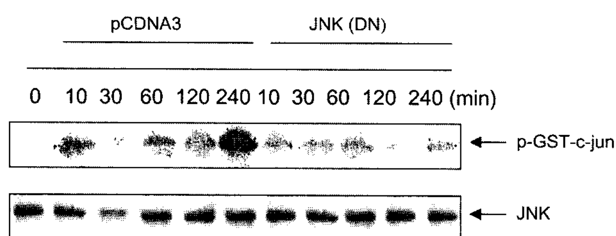

Fig. 3. Nitric oxide induces MAPKs activation in MG-63 osteoblasts. Either pcDNA or JNK (KD)-transfectant cells were exposed to SNAP (0.6 mM) for various times between 0 and 4 h, as indicated. Activation of JNK/SAPK was examined by immunocomplex kinase assays as described in Materials and Methods.

endotoxin and genotoxic stress-activated cascade, and hence may play a pivotal role in determining the apoptosis in MG-63 osteoblasts.

DISCUSSION

NO is a soluble gas in aqueous medium and its biological functions are confined to adjacent cells since its ultrashort half-life limits the availability of newly synthesized NO to substances or cells nearby (Moncada & Higgs, 1999). Recently, it has been reported that pro-inflammatory cytokines such as IL-1β, TNF-α, and IFN-*r* modulate the metabolism of bone remodelling both in vitro and in vivo (Bertolini *et al.*, 1986). These cytokines also induce the production of NO in various cell types of bone tissue. These reports suggest that NO may be a modulator in the cytokine-induced processes of bone remodeling, and especially that it may function in the regulation of bone resorption.

We observed that neither SB203580 (20 μM), a highly specific p38 kinase inhibitor, nor PD98059 (20 μM), a selective inhibitor of MEK1 kinase, had any effect on the apoptosis (Fig. 1B). In contrast, SNAP caused little or no apoptotic phenomena, including caspase-3 activation, in JNK-KD-transfected cells, as compared with that in pcDNA3 only transfected cells. Our present results strongly suggest that JNK/SAPK provide pivotal roles in NO signaling to induce apoptosis, at least in MG-63 human osteoblasts.

The cytotoxic and/or cytostatic actions of NO are not only directed against invading pathogens but are also able to affect susceptible host cells. Therefore, the mechansim of cellular defense that alleviates the damaging potential of these radicals and that account for differential cellular susceptibilities to NO seems very necessary. Protective mechanisms may be attributable to an altered NO-target interaction, scavenging of NO, or efficient repair mechanisms. Our results demonstrate that SNAP induced apoptosis through JNK, caspase-9 and subsequent-3 activation. It has also been reported that cytosolic Cyt-c activates caspase-9, and that the subsequently activated caspase-9 cleavages and activates caspase-3 [32]. In this study, treatment with caspase inhibitory peptides (Z-VAD-FMK, a pan caspase inhibitor, or DEVD-CHO, a caspase-3 inhibitor) abrogated the exogenous NO-induced cell death in human osteoblasts. However, Z-VAD-FMK did not block NO-induced Cyt-c release into the cytoplasm (data not shown). Cyt-c may be an upstream molecule of caspase activation in the NO-induced apoptosis in human osteoblasts. This study showed that JNK inhibition prevented the NO-induced apoptosis.

In conclusion, our results demonstrate that activation of JNK/SAPK is crucial for NO toxicity in MG63 human osteoblasts. These results have possible implications not only for the study of signal pathways involved in NO-induced apoptosis, but also for an understanding of the possible complications of NO synthase induction and of the clinical use of NO donors.

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