NITRIC OXIDE INDUCED OSTEOBLAST APOPTOSIS IS INVOLVED BY THE MODULATION OF INTRACELLULAR CALCIUM CONCENTRATIONS AND CASPASE-3 ACTIVITY

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Introduction: Nitric oxide (NO), a reactive oxidant, is detectable in osteoblasts and contributes to the modulation of bone remodeling (Collin-Osdoby et al., 1995). Our previous study has shown that NO could induce osteoblast apoptosis through the de novo synthesis of apoptotic Bax protein (Chen et al., 2002). Caspases are cystein proteases. The activation of caspases results in the proteolytic cleavage of key cellular targets and then leads to cell death (Gill et al., 2002). Cellular calcium can activate caspase-3 and induces cell apoptosis (He et al., 2002). However, the roles of cellular calcium and caspases in NO-induced osteoblast apoptosis are still unknown. This study is designed to evaluate the effects of NO on intracellular calcium concentrations and caspase-3 activity in osteoblasts using neonatal rat calvarial osteoblasts as the experimental model.

Methods: Osteoblasts were prepared from neonatal Wistar rat calvariae following the sequential enzyme digestion method. Sodium nitroprusside (SNP) was used to be a NO donor. The amounts of nitrite, an oxidative product of NO, in the culture medium of osteoblasts were determined by the Griess reaction method. In order to validate the effect of SNP on the alteration of intracellular calcium concentration, the calcium immobilization within osteoblasts pre-incubated with or without this NO donor was analyzed by using Fluo-3 staining and a fluorescence spectrophotometer (confocal microscope). The immobilization of intracellular calcium was demonstrated by the appearance of “hot spots” within the cytoplasm and perinuclear regions after addition of bradykinin to the cells. The levels of caspase-3 enzymatic activity in osteoblasts were detected by a fluorescent detector. Apoptotic cells were quantified by a flow cytometric method.

Results: Exposure of osteoblasts to SNP concentration- and time-dependingly increased the amounts of nitrite in the culture medium of osteoblasts (data not shown). SNP significantly enhanced the concentrations of intracellular calcium in osteoblasts in a time-dependent manner (Fig. 1). Following the increase in intracellular calcium concentration, administration of osteoblasts with SNP for 2, 4 and 6 hours significantly resulted in 24 %, 2.1-fold and 2.4-fold increases of caspase-3 activity, respectively (Fig. 2). When osteoblasts were exposed to SNP for 4, 8 and 16 hours, the percentages of apoptotic cells were significantly augmented by 4, 7 and 3 folds, respectively (Fig. 3).

Discussion: This study used confocal microscope to show that SNP could enhance the concentrations of intracellular calcium in osteoblasts (Fig. 1). Several lines of evidence reveal that SNP can be decomposed to NO under the presence of biological systems, reducing agents or visible light (Bates et al., 1991). In osteoblasts, our previous study demonstrated that this NO donor could release NO and caused cell insults (Chen et al., 2002). The present study further shows that the augmentation of intracellular calcium concentrations, which was excited by SNP-released NO, might be involved in the NO-induced osteoblast injury.

After administration of SNP, the activities of caspase-3 in osteoblasts were time-dependently increased (Fig. 2). The percentages of apoptotic cells in osteoblasts were also augmented following SNP treatment (Fig. 3). Caspase-3 is a key effector to induce cells undergoing apoptosis (Gill et al., 2002). Thus, caspase-3 might participate in NO-induced osteoblast apoptosis. Previous study had reported that intracellular calcium can activate caspase-3 activity and induce cell apoptosis (He et al., 2002). The events of NO-induced modulations of osteoblast activities in intracellular calcium, caspase-3 activity and apoptotic damage are time-dependent. Therefore, from the present data, we suggest that NO could increase intracellular calcium concentrations first, then activate caspase-3 activity and finally induce osteoblast apoptosis.


Discussion:

![Fig. 1 Fluorescence staining of the intracellular calcium in osteoblasts incubated with Fluo-3 under fluorescence spectrophotometer (confocal microscope). Control and SNP-treated osteoblasts were analyzed before addition of bradykinin (C: 0sec and SNP: 0 sec). After reaction with bradykinin, the distribution of intracellular calcium in control and SNP-treated osteoblasts was continuously detected, and the results at 10 and 30 sec were presented (C and SNP: 10 and 30 sec). Marked enhancement of “hot spots” showed the increased intensity of intracellular calcium. C: control, SNP: sodium nitroprusside.](image1)

![Fig. 2 Effect of SNP on caspase-3 activity in osteoblasts. Osteoblasts were exposed to SNP for 2, 4 and 6 hours. Caspase-3 activity was determined by detecting fluorescent signal after peptide cleavage by this enzyme. Each value was presented as Mean ± SEM for n = 6. * Values significantly different the respective control, P < 0.05.](image2)

![Fig. 3 Effect of SNP on osteoblast apoptosis. Osteoblasts were exposed to SNP for 4, 8 and 16 hours. The percentages of apoptotic cells in osteoblasts were determined by a flow cytometric method. Each value was expressed as Mean ± SEM for n = 6. * Values significantly different from the respective control, P < 0.05.](image3)