MODULATIONS OF INTRACELLULAR OXIDATIVE STRESS, MITOCHONDRIAL MEMBRANE POTENTIAL AND BCL-2/BAX PROTEINS ARE INVOLVED IN NO-INDUCED OSTEOBLAST APOPTOSIS

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Introduction: Nitric oxide (NO) is synthesized by constitutive or inducible NO synthase with various biological activities. NO can regulate bone remodeling. Osteoblasts mediate bone formation. NO has a biphasic role in osteoblast physiology and pathophysiology. Constitutive NO modulates osteoblast growth and differentiation, but overinduction of NO is cytotoxic to the bone cells (Collin-Osdoby et al., 1995). During the inflammation, proinflammatory cytokines and endotoxin lipopolysaccharide stimulate NO production in osteoblasts. Our previous study revealed that NO caused osteoblast apoptosis from analyses of cells' morphologies, apoptotic cells and DNA fragmentation (Chen and Liu, 2000). This study is designed to investigate the modulating effects of NO on intracellular reactive oxygen species (ROS), mitochondrial membrane potential, anti-apoptotic Bcl-2 protein and pro-apoptotic Bax protein using primary neonatal rat calvarial osteoblasts as our 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. NO in culture medium of rat osteoblasts was determined by the Greiss reaction system. Cell viability and ALP activity was analyzed with MTT assay and the Sigma Diagnostics Alkaline, Acid and Prostatic Acid Phosphatase kit, respectively. Intracellular ROS in rat osteoblasts were caught by a 2',7'-dichlorofluorescin diacetate (DCFH-DA) dye and analyzed by a flow cytometer. Mitochondrial membrane potential was analyzed by the flow cytometry method. Cytosolic proteins from untreated and SNP-treated osteoblasts were electrophoretically separated and transferred to nitrocellulose membranes. Immunodetections of Bcl-2 and Bax proteins were carried out using a monoclonal antibody against rat B2-2 protein and a polyclonal antibody against rat Bax protein, respectively.

Results: NO in rat osteoblasts was time-dependently increased following a treatment of 2 mM SNP. Administration of rat osteoblasts with SNP resulted in decreases of ALP activity and cell viability. NO caused osteoblast DNA fragmentation. Intracellular ROS of rat osteoblasts was time-dependently augmented by SNP (Fig. 1). Treatment of rat osteoblasts with SNP lead to the decrease of mitochondrial membrane potential in a time-dependent manner (Fig. 2). SNP inhibited the levels of osteoblast Bcl-2 protein (Fig. 3). Treatment of rat osteoblasts with SNP increased the levels of Bax protein.

Discussion: SNP is a NO donor that is decomposed to NO under the presence of biological tissues or visible light. Administration of rat osteoblasts with SNP increases NO and intracellular oxidative stress. The increase of NO is cytotoxic to osteoblasts. Both of our previous and present data reveal that NO-induced osteoblast death is mainly through an apoptotic pathway (Chen and Liu, 2000). SNP increases intracellular ROS in rat osteoblasts. Because DCFH-DA dye specifically catches peroxides-typed ROS, it is possible that the increase of intracellular oxidative stress in rat osteoblasts treated with SNP is due to the production of peroxynitrite, a combined product of NO and superoxide. SNP decreases osteoblast mitochondrial membrane potential. Alternation of membrane potential in osteoblast mitochondria would cause the failure of ATP synthesis, the increase of cytotoxic oxidants, or the release of apoptotic factors. Bcl-2 is an antiapoptotic protein, but Bax protein is proapoptotic protein. Modulations of intracellular Bcl-2/Bax proteins would drive cells to apoptosis or anti-apoptosis (Brüne et al., 1999). The present study reveals that SNP decreases the level of Bcl-2 protein but increases the level of Bax protein. The effects will result in the decrease of Bcl-2/Bax ratio and drive osteoblasts to apoptosis. From the present data, we propose that the modulations of intracellular oxidative stress, mitochondrial membrane potential, and Bcl-2/Bax proteins would contribute to NO-induced osteoblast apoptosis.