一氧化氮保護骨母細胞免於氧化壓力傷害之分子機制研究:探討 GATA-3/BCL-XL/MITOCHONDRIA 可能扮演的訊息傳遞角色

MOLECULAR MECHANISMS OF NITRIC OXIDE PROTECTION AGAINST OXIDATIVE STRESS-INDUCED OSTEOBLAST APOPTOSIS: SIGNAL-TRANSDUCED ROLES OF GATA-3/BCL-XL/MITOCHONDRIA

中文摘要

骨母細胞(osteoblasts)參與骨頭生成(bone formation)作用，不同系統性(systemic)或局部性(local)因子會調控骨母細胞的活性，一氧化氮(nitric oxide; NO)即爲此種因子之一。先前研究已證實，NO 前處理(NO precondition)可以保護肝臟細胞、心肌細胞、內皮細胞和神經細胞免於不同壓力(stress)的傷害。而在本實驗室之前系列的研究結果顯示，骨母細胞在發炎(inflammation)狀態下，會因氧化壓力(oxidative stress)的上升進而導致細胞功能喪失(dysfunction)或死亡(death)。因此，本研究想進一步探討，NO 前處理是否可以保護骨母細胞免於氧化壓力所造成的傷害，以及其可能發生的分子機轉。

本研究先前實驗結果顯示，骨母細胞處理低濃度 0.3 mM sodium nitroprusside (SNP)，會少量增加細胞 NO 的生成，但不會造成細胞的損傷，但是，當 SNP 處理的濃度達到 2 mM 時，則會明顯增加細胞內活氧物質(reactive oxygen species)的量，並使細胞受到傷害。因此，本研究將分別處理骨母細胞 0.3 和 2 mM 的 SNP，以當作 NO 和氧化壓力的來源。

骨母細胞經氧化壓力處理後，細胞型態會產生皺縮(shrinkage)的現象，而鹼性磷酸酶(alkaline phosphatase activity; ALP)活性和細胞存活率亦會降低，然而，當骨母細胞前處理 NO 作用 24 小時後，則會明顯改善氧化壓力所造成的細胞型態改變，並回復 ALP 活性和提升細胞存活率。氧化壓力會透過細胞凋亡機制，進而造成骨母細胞死亡，而 NO 的前處理則可以保護細胞免於此種毒性作用。當骨母細胞遭受氧化壓力傷害時，粒線體膜電位(mitochondrial membrane potential)會明顯降低，而前處理 NO 則可以回復此種抑制作用。骨母細胞 Bax 蛋白的生合成，也會因氧化壓力作用而增加，進而引發 cytochrome c 由粒線體釋放。然而，NO 前處理則可以降低氧化壓力所誘導 Bax 與 cytochorme c 蛋白增加的情形。隨著細胞質 cytochorme c 量的上升，caspase-3 酵素活性也會隨著氧化壓力的處理而增加，並誘導 DNA 發生片斷性損傷(DNA fragmentation)。但前處理 NO，則可以減緩氧化壓力所造成的細胞傷害。由以上實驗結果顯示，NO 可以保護骨母細胞免於氧化壓力的傷害，而其可能發生的保護機轉，是透過抑制粒線體相關的凋亡路徑(mitochondria-dependent apoptotic pathway)。

爲了進一步闡明細胞相關凋亡蛋白(pro- and anti-apoptotic proteins)於 NO 保護作用中可能扮演的角色，本研究進一步探討，凋亡蛋白 Bax 與抗凋亡蛋白 Bcl-XL 在骨母細胞中的轉位作用(translocation)和基因表現(gene expression)。實驗結果顯示，氧化壓力會活化 Bax 蛋白，使其由細胞質轉位至粒線體。相反地，氧化壓力卻會降低 Bcl-XL 的轉位現象。然而，經 NO 前處理後，則明顯改善氧化壓力對此二種蛋白的轉位作用。進一步由 RNA 和蛋白分析結果發現，氧化壓力會增加骨母細胞 Bax 蛋白量，但不會影響其 mRNA 的生合成。NO 的前處理不會改變細胞 Bax mRNA 和蛋白的量，但可以減少氧化壓力所引發 Bax 增加的情形。與 Bax 相較之下，氧化壓力則會抑制細胞 Bcl-XL mRNA 和蛋白的量，NO 的前處理則會明顯回復 Bcl-XL mRNA 與蛋白的生合成。

本研究緊接著探討 NO 對於轉錄因子(transcriptional factor) GATA-3 的影響，以進一步闡明調控 bcl-xl 基因表現之分子機制。氧化壓力會抑制 GATA-3 RNA 和蛋白的表現，而 NO 前處理則會回復此一作用。由 electrophoretic mobility shift assay 分析結果發現，氧化壓力會降低骨母細胞 GATA-3 核蛋白與其特定之 DNA 序列(GATA-3 DNA element)的鍵結(transactivation)，但此種抑制作用會因 NO 前處理而得到減緩。爲進一步說明 GATA-3 是否參與 bcl-xL 的基因調控，以 GATA-3 siRNA 處理骨母細胞後發現，細胞內 GATA-3 蛋白量會隨之降低。同時，GATA-3 siRNA 的處理，亦會抑制 NO 所誘導 Bcl-XL mRNA 的回復作用。因此，隨著 GATA-3 siRNA 的處理，會導致骨母細胞 GATA-3 和 bcl-xL 的表現降低，進一步抑制 NO 對骨母細胞的保護作用。

綜合以上結果，本研究發現 NO 前處理可以保護骨母細胞免於氧化壓力所誘導的凋亡傷害，其作用機轉是透過 GATA-3 所調控 bcl-xL 基因的表現，經由活化 Bcl-XL/mitochondria/caspase protease 的路徑。由此一研究結果，將更能闡明 NO 對於活化骨母細胞活性過程中，可能扮演的分子調控角色，並有助於往後骨頭相關疾病的預防與治療。

英文摘要

Osteoblasts mediate bone formation. There are varieties of systemic and local factors participating in regulation of osteoblast activities. Nitric oxide (NO) is one of such critical factors. Previous studies have shown that NO precondition can protect hepatocytes, cardiomyocytes, endothelial cells, and neural cells from stress-induced insults. Meanwhile, the effects of NO on oxidative stress-induced injuries are still unknown. Therefore, this study was aimed to evaluate the protective effects of NO pretreatment on oxidative stress-induced osteoblast apoptosis and its possible signal-transducing mechanisms using neonatal rat calvarial osteoblasts as the experimental model.

Our results revealed that exposure of osteoblasts to sodium nitroprusside (SNP) at a

low concentration of 0.3 mM increased cellular NO levels without affecting cell viability. However, when the concentration reached a high concentration of 2 mM, SNP increased the levels of intracellular reactive oxygen species and induced osteoblast injuries. Thus, administration of 0.3 and 2 mM SNP in osteoblasts was respectively used as sources of NO and oxidative stress.

Pretreatment with NO for 24 h ameliorated the oxidative stress-caused morphological alterations and alkaline phosphatase activity decreases, and reduced cell death in osteoblasts. Oxidative stress induced osteoblast death via an apoptotic mechanism, but NO pretreatment protected osteoblasts against the toxic effects. The mitochondrial membrane potential was reduced following exposure to the oxidative stress. However, pretreatment with NO significantly lowered the suppressive effects. Oxidative stress increased cellular Bax protein production and cytochrome c release from mitochondria. Pretreatment with NO decreased oxidative stress-caused augmentation of Bax and cytochrome c protein levels. In parallel with cytochrome c release, oxidative stress induced caspase-3 activation and DNA fragmentation. Pretreatment with NO reduced the oxidative stress-enhanced caspase-3 activation and DNA damage. Results of this study show that NO pretreatment can protect osteoblasts from oxidative stress-induced apoptotic insults. The protective action involves a mitochondria-dependent mechanism.

To further evaluate the molecular mechanisms of NO's protection against oxidative stress-induced apoptotic insults to osteoblasts, the protein translocation of Bax and Bcl-XL from the cytoplasm to mitochondria and their gene expressions were analyzed. The data showed oxidative stress increased the translocation of Bax protein from the cytoplasm to mitochondria but decreased Bcl-XL's translocation. Pretreatment with NO ameliorated the oxidative stress-induced modulation in translocation of Bax and Bcl-XL. RT-PCR and immunoblot analyses revealed that oxidative stress increased cellular Bax protein levels but did not affect the mRNA synthesis. Pretreatment with NO did not change Bax mRNA and protein syntheses but significantly lowered oxidative stress-caused increases in the protein levels. In comparison, oxidative stress decreased the levels of Bcl-XL mRNA and protein. After NO pretreatment, the oxidative stress-induced suppression in productions of Bcl-XL mRNA and protein reversed.

In parallel with the effects of NO and oxidative stress on Bcl-XL expression, the synthesis of transcriptional factor GATA-3 mRNA and its nuclear level were decreased after administration of oxidative stress. Pretreatment with NO alleviated the suppressive effects. The results from EMSA analysis revealed that oxidative stress decreased the transactivation of nuclear extracts binding to GATA-3 DNA elements. Pretreatment with NO significantly lowered the suppressive effect. Application of

GATA-3 siRNA decreased cellular GATA-3 protein levels and ameliorated NO-induced Bcl-XL mRNA synthesis. The NO-involved protection could be decreased after GATA-3 siRNA administration.

Taken together, this study shows that pretreatment with NO can protect osteoblasts from oxidative stress-induced apoptotic insults through regulation of GATA-3-mediated bcl-xL gene expression as well as activation of the Bcl-XL/mitochondria/caspase protease pathway. After execution of this study, we have understood more about the molecular mechanisms of NO-participated regulation of osteoblast activities. This study is helpful to prevention and treatment of bone diseases.