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Nasunin, a new player in the field of osteoblast protection against oxidative stress

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ABSTRACT

This study investigated whether nasunin, the major component of anthocyanin pigment of eggplant (*Solanum melongena* L.), known for its antioxidant effects, counteracts *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative damage in MC3T3-E1 osteoblastic cells. Pretreatment with purified nasunin (10^{-10}–10^{-6} M) significantly increased viability and reduced apoptosis of MC3T3-E1 cells cultured with *t*-BHP (250 µM, 3 h). Furthermore, nasunin (10^{-9} M) prevented *t*-BHP-induced osteoblastic dysfunction and changes in the cytoskeleton organisation due to both an increase of intracellular glutathione and a decrease of intracellular reactive oxygen species. Moreover, *t*-BHP-induced reduction in osteoblast differentiation markers, such as alkaline phosphatase and collagen content, was recovered in nasunin-pretreated cells. Nasunin protects against *t*-BHP-induced osteoblastic dysfunction by activating the phosphoinositide 3-kinase/Akt signalling pathway (PI3K/Akt) since LY294002, a PI3K-specific inhibitor, worsened the cytotoxic effect of *t*-BHP and reversed the protective action of nasunin. Furthermore, nasunin prevented the decreased Akt phosphorylation observed in *t*-BHP-treated cells.

1. Introduction

During adult life bone structure and function are maintained through a process termed remodelling. Bone remodelling involves old bone resorption by osteoclasts and synthesis of new bone in its place by osteoblasts into temporary anatomic structures called basic multicellular units (BMU).

The maintenance of bone homeostasis depends on the balance of cellular activities during bone remodelling. Negative BMU balance, due to a persistent excess of bone resorption over formation, results in a number of disease conditions including osteoporosis (McCormick, 2007). Osteoporosis is a skeletal disease characterised by low bone mass and compromised bone strength leading to a deterioration of microarchitecture, which predisposes to fractures resulting from minimal trauma insufficient to fracture normal bone.

Several interacting factors contribute to the risk of osteoporotic fracture, including hormonal, behavioural, nutritional and genetic variables (Post, Cremers, Kerbusch, & Danhof, 2010).

Recent epidemiological evidence in humans and experimental studies in rodents indicate that oxidative stress induced

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by reactive oxygen species (ROS) plays an important role in the development of osteoporosis. ROS are generated by the mitochondrial electron transport chain during normal metabolism. Progressive mitochondrial damage with increasing age may result in excessive ROS production, which damages proteins, lipids and DNA leading to cell death (Balaban, Nemoto, & Finkel, 2005).

Pharmacological and genetic studies in mice reported a negative effect of oxidative stress on bone mass. Treatment with antioxidants reduces gonadectomy-induced bone loss (Almeida et al., 2007). Mice with deletion of the antioxidant gene Sod1 show low bone mass, which worsens with age (Nojiri et al., 2011). Furthermore, a link between oxidative stress and a decrease in bone mineral density (Altindag, Erel, Soran, Celik, & Selek, 2008) as well as a protective effect of antioxidants on bone resorption (Sanders, Kotowicz, & Nicholson, 2007) has been reported in several clinical studies.

At cellular level, ROS increase osteoclast number and activity by increasing receptor activator of NF-κB ligand (RANKL) expression in osteoblasts. ROS greatly influence the generation and survival of osteoblasts and osteocytes, former osteoblasts that are entombed in the mineralised matrix and are responsible for sensing and adapting bone to mechanical loading (Almeida & O'Brien, 2013).

Among the various non-pharmacological measures that improve or maintain bone health, recent studies have focussed on nutritional strategies that include not only an adequate intake of calcium, other minerals and proteins but also food rich in antioxidants. Vegetables are rich in phytonutrients and are especially known for their scavenging action against the free radicals generated by oxidative stress (Wojcik, Burzynska-Pedziwiatr, & Wozniak, 2010).

Diets high in fruits and vegetables, and thus, rich in dietary antioxidants deserve a central place in the assessment and management of a variety of age-related diseases including osteoporosis. Experimental studies have shown skeletal benefits of isolated nutrients such as flavonoids, lycopene and resveratrol. Furthermore, dietary antioxidant intake seems to exert a beneficial effect on bone metabolism and mineral density in postmenopausal women (Sacco, Horcajada, & Offord, 2013).

With the aim of studying the effects of vegetables with antioxidant activities on bone cells, we focused our attention on the extracts of eggplant (*Solanum melongena* L.). Eggplant, in fact, is ranked among the top ten vegetables in terms of oxygen radical absorbance capacity (ORAC) since it is particularly rich in antioxidants belonging to polyphenols (Cao, Sofic, & Prior, 1996).

Among the various part of eggplant (entire fruit, pulp, skin), peeled skin possess a high capacity in the scavenging of superoxide radicals and contains the higher amount of anthocyanin pigment of eggplant (Kayamori & Igarashi, 1994). It has been reported that the major anthocyanins in eggplant are delphinidin-3-rutinoside, and *trans*- and *cis*-delphinidin 3-(*p*-coumaroylrutinoside)-5-glucoside (nasunin), being the *trans*-isomer the largely present in natural extracts, with an average value of 587 μmol/100 g, dw detected in Japanese Nasunin-type eggplants (Mennella et al., 2012). Among several anthocyanins, delphinidin glucosides exert the strongest scavenging activity against superoxide anion and peroxynitrite (Rahman, Ichiyanagi, Komiyama, Hatano, & Konishi, 2006). *In vitro* studies showed nasunin antioxidant activities expressed by its capacity to scavenge superoxide anion and to inhibit lipid peroxidation (Noda, Kneyuki, Igarashi, Mori, & Packer, 2000; Salerno et al., 2014). *In vivo* studies was found that dietary nasunin has a cholesterol-lowering effect (Kayamori & Igarashi, 1994) and protects against paraquat-induced oxidative stress (Kimura, Araki, Takenaka, & Igarashi, 1999).

The role of nasunin, if any, in the protection against oxidative stress in osteoblasts has never been investigated. Considering that ROS have adverse effects on the osteoblast phenotype and function (Lee, Lim, Lee, & Yang, 2006), in the present study, we examined the effects of *trans*- isomer of nasunin, purified by crystallisation, on the proliferation and differentiation of MC3T3-E1 osteoblast-like cells under oxidative challenge. We used MC3T3-E1 osteoblast like cells considered a suitable model for studying osteogenic development *in vitro*. These cells, in fact, are characterised by distinct proliferative and differentiation stages, thereby reproducing a temporal programme similar to osteoblast differentiation as occurs during *in vivo* formation (Sudo, Kodama, Amagai, Yamamoto, & Kasai, 1983). To induce oxidative damage, we used *t*-BHP, which is an organic hydroperoxide widely used as a prooxidant in several cells (Altman et al., 1994) including MC3T3-E1 osteoblastic-like cells (Dieci, Casati, Pagani, Celotti, & Sibilia, 2014). On the basis of the results obtained, showing a protective effect of nasunin against *t*-BHP-induced oxidative damage, we examined the molecular pathways involved in such nasunin action in MC3T3-E1 cells.

2. Materials and methods

2.1. *Chemicals*

Tert-butylhydroperoxide (*t*-BHP), Trolox and LY294002 were purchased from Sigma-Aldrich (Milano, Italy). Trolox was dissolved in ethanol (70%) with a final ethanol concentration in the medium less than 0.5%.

2.2. *Nasunin purification by crystallisation*

Eggplant fruits (*Solanum melongena* L.), from several genotypes of Japanese-type cultivars of Italian origin belonging to the "Tunisina" typology, characterised by nasunin presence in the peel (Mennella et al., 2012), were harvested at the commercial ripening stage. The plants were grown and harvested at commercial ripening stage during the 2011 season in an experimental field at CREA – Unità di Ricerca per l'Orticoltura, located in Montanaso Lombardo (Lodi, Italy). Once harvested, eggplant fruits were immediately taken to the laboratory and the peels were carefully separated from the flesh with a lancet and were quickly frozen in an air-blast tunnel at −50 °C. The frozen material was then lyophilised and reduced to a powder with the same particle size by passing through a 1 mm diameter sieve, and stored at −20 °C. Ten grams of powder was extracted with 500 mL of 0.03 M HCl, by vigorous stirring for one hour at room temperature. The mixture was then centrifuged at 25000 × *g* at 4 °C for 20 min and the supernatant was