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Hydrogen peroxide inhibits formation of cartilage in chicken micromass cultures and decreases the activity of calcineurin: implication of ERK1/2 and Sox9 pathways

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Abstract

Calcineurin was found as a positive regulator of chondrogenesis in chondrifying chicken micromass cultures (HDCs), as cyclosporine A (CsA) reduced both the amount of cartilage and the expression of mRNAs of aggrecan and the chondrogenic transcription factor Sox9. Cartilage formation was inhibited by H_2O_2 in a concentration-dependent manner without loss of cellular viability or severe decrease of cell number. Expression of both the mRNA and the unphosphorylated protein Sox9 was decreased, while its phosphorylation was stimulated by either H_2O_2 or CsA. Oxidative stress decreased the activity of calcineurin but the phosphorylation of the member of MAPK family ERK1/2 was extremely elevated either by 1 mM H_2O_2 or 2 μM CSA. The ERK inhibitor PD098059 attenuated the depletion of cartilage matrix as well as decreased the expression and phosphorylation of Sox9 in cultures treated with H_2O_2 or CsA. Our results suggest that the chondrogenesis-inhibiting effect of H_2O_2 is mediated, at least partly, by inhibition of calcineurin and by activation of ERK1/2. We also propose a regulatory role of calcineurin in the phosphorylation level of either ERK1/2 or Sox9 and a positive role of ERK1/2 in regulating both the expression level and the phosphorylation state of Sox9 in chicken HDCs.

Keywords: Chondrogenesis; Oxidative stress; Protein phosphorylation; Protein phosphatases; Calcineurin; Cyclosporine A; ERK1/2; PD098059; Sox9

Introduction

The differentiation of mesenchymal cells into chondrocytes is a multistep process including the recruitment of mesenchymal progenitor cells, the subsequent condensation of these cells, followed by the differentiation of the condensed mesenchymal cells into chondrocytes. At this stage, the cells surround themselves with an abundant layer of extracellular matrix and start to express cartilage-specific extracellular matrix molecules such as collagen type II and aggrecan [1]. Expression of type II collagen and core protein of aggrecan is controlled by Sox9, one of the major transcription factors regulating cartilage differentiation. Sox9 is a high-mobility-group domain containing transcription factor expressed in all chondrocyte progenitors and chondrocytes [2–4]. The activity of Sox9 protein is regulated by phosphorylation of a serine 211 residue which is a target of cAMP-dependent protein kinase (PKA) [5]. Chondrogenesis is controlled or influenced by many diverse signals including TGFβ and BMPs [6], PKA [7], and PKC/MAPK [8,9] pathways. Most of these pathways involve various protein kinases in the regulation of

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chondrogenesis, but little is known about the function of protein phosphatases in this process. Our group has given the first evidence of the possible involvement of one of the major cellular phosphatases, PP2A, in the regulation of PKA/CREB axis during chondrogenesis [10]. Recently, the role of another protein phosphatase PP2B, also named as calcineurin, in the regulation of cartilage formation has been described [11].

Calcineurin is a phosphoserine/phosphothreonine-specific protein phosphatase consisting of a 59-kDa catalytic subunit (calcineurin A) and a smaller 19-kDa Ca^{2+}-binding regulatory subunit (calcineurin B). The classical mechanism by which calcineurin is regulated in vivo is via changes in the intracellular Ca^{2+} concentration. Since in resting cells the cytoplasmic Ca^{2+} concentration is low, calcineurin exists chiefly in an inactive form (for a review, see Ref. [12]). The elevated intracellular calcium can induce chondrogenesis through the calcineurin/NFAT signaling axis that activates BMP2 expression [11]. A number of calcineurin inhibitors have been isolated, the most potent, specific, and well-known compounds are the immunosuppressant drugs cyclosporine A (CsA) and FK506 (tacronimus) which inhibit calcineurin when complexed with their respective cytoplasmic receptors cyclophilin and FKBP (FK506 binding protein). Recent studies have indicated that both CsA and FK506 also inhibit members of mitogen-activated protein kinases (MAPKs) pathway in T lymphocytes [13,14].

MAPKs are responsible for the conversion of a large number of extracellular stimuli into cellular responses that range from positive and negative roles in cell proliferation, differentiation, and apoptosis to regulation of inflammatory and stress responses. All of the MAPK pathways are organized into cascades that work in series to result in simultaneous phosphorylation of tyrosine and serine residues within the conserved Thr-X-Tyr motif in the activation loop of the kinase domain [15]. MAPKs are phosphorylated by dual-specific MAPK kinases while inactivation occurs via the activity of MAPK phosphatases. Both MAPK kinases [16] and phosphatases [17] are regulated by many diverse signaling pathways. The MAPK superfamily is made up of three main and distinct signaling pathways: ERK1/2, p38, and JNK. The latter two are chiefly activated in response to cellular stresses, while ERK1/2 is widely accepted as one of the major mediator of mitogenic extracellular stimuli and also plays a role in the differentiation of several cell types [15].

It is known that MAPKs have crucial roles in the fate of chondrogenic cells. p38 stimulates while ERK1/2 inhibits formation of cartilage, and this pathway is Ca^{2+}-sensitive due to the involvement of PKC [18]. Also, there are accumulating data on the role of calcineurin in the regulation of the activity of MAPKs [19,20], but there is evidence for the opposite relation of the two mechanisms as well, a role of the p38 pathway in the nuclear shuttling of NFAT-2 [21]. Although calcineurin is declared as one of the major mediator of the signals elevating intracellular Ca^{2+} level, but recently a further mechanism for regulating calcineurin has been discovered. This involves redox reactions of active site metal ions, and it has been described that superoxide dismutase protects calcineurin from inactivation [22]. The activity of calcineurin is affected by extracellular oxidants, in particular H_2O_2 [23,24].

Reactive oxygen species (ROS), including H_2O_2, have been presented as intracellular agents influencing various signal transduction mechanisms. ROS stimulate tyrosine phosphorylation by the activation of several kinases, such as proteins of the MAPK pathway and Src family [25,26]. One mechanism by which hydrogen peroxide regulates cellular processes is the transient inhibition of protein tyrosine phosphatases through the reversible oxidation of their catalytic cysteine, which suppresses protein dephosphorylation [27]. Calcineurin is also a candidate for redox regulation, the inactivation of calcineurin by hydrogen peroxide is probably due to a bridging of two closely spaced Cys residues [28]. It was also demonstrated that the intracellular calcineurin activity is sensitive to the inactivation by hydrogen peroxide, with an IC_{50} of 30–40 μM [29]. ROS affect diverse cellular functions of articular tissues (for a review, see Ref. [30]). Additionally, isolated articular chondrocytes are capable to produce H_2O_2 either under physiological conditions or following anoxia-reoxygenation [31], and H_2O_2 is described to inhibit proteoglycan synthesis of cultured bovine articular chondrocytes [32]. Hypoxic conditions also generate free radicals in inflamed synovial tissues [33,34]. These findings describe the effects of free radicals on mature, differentiated tissues of joints; however, little is known about the effect of ROS on the regeneration or on the de novo formation of cartilage. Since during inflammatory diseases of articular cartilage a number of ROS are liberated [35,36], it can be a subject of interest how these compounds influence the possible renewal of damaged cartilage [30]. To elucidate this, we investigated the effects of oxidative stress on the formation of cartilage in high density micromass cell cultures of embryonic chondrogenic cells of chicken.

Here we give evidence for the role of calcineurin in the regulation of chondrogenesis under normal conditions and during oxidative stress. We found that oxidative stress inhibited chondrogenesis and the activity of calcineurin was reduced. Oxidative stress also caused hyperphosphorylation of ERK1/2 both on Ser/Thr and Tyr residues, and we propose a regulatory role of calcineurin in the control of the phosphorylation level of ERK1/2 either in normal chondrogenesis or under oxidative stress. Involvement of Sox9 transcription factor is also discussed.

Materials and methods

Cell culturing

Distal parts of the limb buds of Ross hybrid chicken embryos of Hamburger–Hamilton stages 22–24 were