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Inhibition of extracellular matrix production and remodeling by doxycycline in smooth muscle cells

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Alterations in the extracellular matrix (ECM) production and remodeling of smooth muscle cells (SMCs) have been implicated in processes related to the differentiation in atherosclerosis. Due to the anti-atherosclerotic properties of the tetracyclines, we aimed to investigate whether cholesterol supplementation changes the effect of doxycycline over the ECM proteins synthesis and whether isoprenylated proteins and Rho A protein activation are affected. *SMC* primary culture isolated from chicks exposed to atherogenic factors *in vivo* (a cholesterol-rich diet, SMC-Ch), comparing it with control cultures isolated after a standard diet (SMC-C). After treatment with 20 nM doxycycline, [H^3]-proline and [H^3]-mevalonate incorporation were used to measure the synthesis of collagen and isoprenylated proteins, respectively. Real-time PCR was assessed to determine *col1a2*, *col2a1*, *col3a1*, *fibronectin*, and *mmp2* gene expression and the pull-down technique was applied to determine the Rho A activation state. A higher synthesis of collagens and isoprenylated proteins in SMC-Ch than in SMC-C was determined showing that doxycycline inhibits ECM production and remodeling in both SMC types of cultures. Moreover, preliminary results about the effect of doxycycline on protein isoprenylation and Rho A protein activation led us to discuss the possibility that membrane G-protein activation pathways could mediate the molecular mechanism.

1. Introduction

Tetracyclines have anti-atherosclerotic properties that are not inherent to their bactericidal activity (1). Thus, they inhibit matrix metalloproteinase (MMP) activity and are used to treat diseases that involve tissue destruction (2). These compounds also inhibit matrix degradation of abdominal aortic aneurysms (1,3), suppress cellular proliferation and migration (4,5) and the synthesis of extracellular matrix (ECM) and intimal hyperplasia (6,7). These inhibitory effects have been attributed to the capacity of the tetracyclines to chelate divalent ions, but this only explains in part their inhibition of MMP activity due to the sequestering of the Zn^{2+} needed for their function (1). Several studies have demonstrated that tetracyclines inhibit MAPK and PI3K signaling pathways (5,8), closely related to G proteins and membranes. These are two of the most important signaling pathways in the development of atherosclerosis and may account for the tetracyclines mechanism of action in these processes, which has yet to be well characterized and is one of the goals of this paper.

The signaling pathways involved in atherosclerosis include isoprenylated proteins and small GTPases. A subfamily of these proteins is Rho, which plays a key role in signaling pathways that control proliferation, apoptosis, matrix adhesion and cell migration (9–12). These processes are important for the physiology and normal development of individuals and any deregulation of the pathways in which these proteins are involved leads to a multitude of pathological processes, including atherosclerosis (13). In smooth muscle cell (SMC) contraction, Rho A is recognized as the main regulator of the calcium sensitization of contractile proteins (14)

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and as a critical protein in SMC differentiation control through regulation of serum-response factor (SRF)-dependent transcription (15). SMC differentiation is marked by the expression of cytoskeletal and contractile proteins, coordinated by SRF (15), whose cell localization is regulated by Rho A, which thereby modifies the expression of genes involved in cell differentiation (16). Various studies have demonstrated that Rho A inhibition reduces the expression of SMC differentiation marker genes (17).

In our laboratory, we are investigating changes in the ECM of SMC in processes related to atherosclerosis, focusing on the effects of cholesterol feeding on the expression of the major regulating genes (18). In the present gene expression analysis, we used a chick SMC primary culture model in which SMC were exposed to atherogenic factors *in vivo* (cholesterol-rich diet, SMC-Ch), comparing these cultures with control ones isolated after a standard diet (SMC-C) (19). Our objective was to investigate the effects of doxycycline on ECM production and remodeling in SMC-Ch and SMC-C cultures as well as on isoprenylated proteins synthesis and Rho A activation.

2. Materials and methods

2.1. Animals and treatment

The protocol for this study was approved by the University of Granada (Spain) Animal Laboratory Service and chicks received humane treatment in compliance with the Animal Research regulations of the European Union. Newborn White Leghorn male chicks ("Gallus domesticus") were supplied by the same service. The diet was started at hatching and maintained until the chicks were killed with food and water available *ad libitum*. Two groups of 10-day-old chicks were used: control diet group (C-group) kept on a standard diet (Sanders A-00), and treated group (Ch-group) fed on the same diet homogeneously mixed with 5% w/w powdered cholesterol (Panreac reagent Barcelona, pure grade). After the treatments, animals were anesthetized with ketamine (60 mg kg^{-1} of body weight) and sodium pentobarbital (50 mg kg^{-1} of body weight) in compliance with the European Union Animal Research rules.

2.2. SMC separation and culture

SMC were isolated from the aortic arch of the chicks, as previously described (20,21) with slight modifications (19) and cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with D-glucose (4.5 g/l), L-glutamate (0.584 g/ml) (Flow), antibiotic cocktail composed of penicillin (100 μg/ml) and amphotericin (0.25 μg/ml) (Sigma), and 10% (v/v) fetal bovine serum (FBS). Media were buffered with bicarbonate and cultures were kept at 37 °C in humidified atmosphere (95% air/5% CO_2). Media were renewed three times weekly. Secondary cultures were initiated after low or high passages using 0.05%/0.02% Trypsin-EDTA solution. All experiments were conducted using 3 or 4 passages. Cells were identified as vascular SMC by their hill-and-valley configuration at confluence (21) and their positive fluorescence staining for smooth muscle actin and myosin (22). SMC cultures obtained from control-diet fed chicks were designated as SMC-C, and SMC cultures obtained from chicks fed with the control diet supplemented with 5% cholesterol as SMC-Ch.

2.3. In vitro treatments of cultures

All *in vitro* treatments were performed in SMC cultures at 90% confluence, when ECM synthesis in SMC started to be evident. Doxycycline (D9891, Sigma) was diluted in culture medium at a concentration of 10 μg ml^{-1} (20 nM), at which no toxicity or variation in primary cultured SMC proliferation has been reported, as well as in other cell lines (23–25) and the incubation time was 48 h.

2.3.1. [H^3]-proline incorporation

The amount of SMC-synthesized collagen was determined by the Peterkofsky method (26). SMC-C and SMC-Ch were seeded at equal cell density in 6-well plates and, when confluence reached 90%, 1 ml culture medium was added to each well containing $3.7 \cdot 104$ Bq L-[5-H^3]-proline ($9.62 \cdot 10^{11}$ Bq/mmol) (Amersham, Buckinghamshire, UK). After 48 h incubation, cells were lysed with 0.5 ml 0.5 M NaOH for 1 h. The resulting solution was neutralized with an equal amount of 0.5 M HCl, and 50 μl were used to measure total proteins with the Bradford method (27). One volume of 10% TCA was added to the remaining 250 μl and centrifuged at 13,000 g for 15 min at 4 °C. The resulting precipitate was dissolved in 100 μl 0.2 M NaOH, and then neutralized with 1 M HCl. The solution was incubated with collagenase buffer (Tris–HCl, pH 7.6 20 mM, and $CaCl_2$ 250 mM final concentration) and 10 units of collagenase (Sigma) at 37 °C overnight. Then, 150 μl 10% TCA were added and centrifuged at 13000 g for 15 min at 4 °C. The resulting supernatant was added to 4 ml of scintillation fluid (Cocktail Biogreen 3 for liquid scintillation, Scharlau) and the radioactivity was measured in a liquid scintillation counter LS 600 TA (Beckman Instruments).

2.3.2. [H^3]-mevalonate incorporation

SMC and SMC-Ch were seeded in 12-well culture plates at equal cell density. When cells reached 90% confluence, the medium was removed and 1 ml fresh culture medium containing $3.7 \cdot 10^4$ Bq [H^3]-mevalonate was added to each well. [H^3]-mevalonate was obtained from R,S-[2-H^3] mevalonolactone ($4.26 \cdot 10^{10}$ Bq/mmol) (Amersham). The process involved evaporating tritiated mevalonolactone to dryness, hydrolyzing with NaOH 0.1 M at 37 °C for 1 h and neutralizing it with 0.1 M HCl and Hepes 100 mM pH 7.4. It was then immediately used for the experiment. After the 48 h incubation with tritiated mevalonate, cells were lysed with 200 μl 0.1 M NaOH at 4 °C overnight. A 100 μl aliquot was used to measure total proteins with the Bradford method (27); 4 ml of scintillation fluid (*Cocktail Biogreen 3 for liquid scintillation*, Scharlau) were added to the remaining 100 μl and radioactivity was measured in an LS 600 TA liquid scintillation counter (Beckman Instruments).

2.4. Real-time PCR analysis

Total RNA from primary SMC cells cultures was isolated with TRIZOL reagent (Invitrogen). Single-stranded cDNA was synthesized from 4 μg total RNA using Oligo (dT)12–18 and PowerScript™ reverse transcriptase (Clontech) in a final reaction volume of 20 μL. Real-time PCR was performed with the Fast Start DNA Master SYBR Green I Kit (Roche) and Chromo4 PCR Detection System (Biorad). For the Light Cycler reaction, a master mix of the following reaction components was prepared to the indicated final concentration: 12.6 μl H_2O, 2.4 μl $MgCl_2$ (4 mM), 1 μl forward primer (0.5 μM), 1 μl reverse primer (0.5 μM), and 2.0 μl of the Fast Start DNA Master SYBR Green I mix (Roche Diagnostic). 1 μl cDNA was added to 19 μl Light Cycler master mix as PCR template. Table 1 contains the primer sequences used in this study, optimized to an equal annealing temperature of 55 °C. Light Cycler products of the different gene expressions were analyzed by agarose gel electrophoresis, and a Light Cycler melting curve was constructed to test for a single product at the end of each PCR reaction. Real-time PCR efficiencies were evaluated by using different starting amounts of cDNA for gapdh, col1a2, col2a1, col3a1, fibronectin, and mmp2 genes. These cDNAs were diluted ($2.5 \cdot 10^{-1}$; $5.0 \cdot 10^{-2}$; $1 \cdot 10^{-2}$; $2 \cdot 10^{-3}$; $4 \cdot 10^{-4}$; $8 \cdot 10^{-5}$) and amplified. A mathematical model

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