Histamine is involved in the regulation of collagen content in cultured heart myofibroblasts via H_2, H_3 and H_4 histamine receptors

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Received November 23, 2020; Accepted June 7, 2021

DOI: 10.3892/br.2021.1447

Abstract. Histamine is involved in the regulation of collagen metabolism during healing following a myocardial infarction; however, its effects on the intact heart tissue is unknown. The aim of the present study was to determine whether histamine may influence collagen content in cells isolated from intact heart, and to identify the histamine receptor involved in the regulation of collagen deposition. Cells were isolated from intact rat hearts and subjected to identification by flow cytometry. The effects of histamine and its receptor agonists and antagonists were investigated. The heart cells were found to be actin, desmin and vimentin positive. Histamine (used at a concentrations of $1x10^{-10}$-$1x10^{-5}$ M) increased collagen content within the culture and increased the expression of α1 chain of the procollagen type III gene. The H_2, H_3 and H_4 receptor inhibitors ranitidine, ciproxifan and JNJ 7777120 blocked the effect of histamine on collagen content. All tested histamine receptor agonists, *viz*. 2-pyridylethylamine dihydrochloride (H_1 receptor agonist), amthamine dihydrobromide (H_2 receptor agonist), imetit (H_3 receptor agonist) and 4-methylhistamine hydrochloride (H_4 receptor agonist), elevated collagen content within the heart myofibroblast cultures. The cells isolated from the intact heart were identified as myofibroblasts. Thus, the results of the present study showed that histamine augmented collagen content in the heart myofibroblast culture by activation of three histamine receptors (H_2, H_3 and H_4). The effect of the amine was also dependent on the activation of collagen type III gene expression.

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Key words: collagen, cell culture, heart, histamine, histamine receptor, myofibroblast

Introduction

Homeostasis of the extracellular matrix within the heart is dependent on cardiac fibroblasts, which account for 60-70% of cells in the human heart. These cells are responsible for the synthesis and breakdown of the extracellular matrix within the heart (1,2). They are also able to detect physical and biological stimuli; these stimuli may change the activity of the cardiac fibroblasts and their potential for collagen synthesis (2,3). It has been found that heart fibroblasts secrete cytokines with autocrine or paracrine regulatory effects (2,3): Fibroblasts isolated from the heart of a patient with heart failure were found to release higher levels of cytokines under inflammatory conditions, and this effect was independent of hypoxia (3). The bioactive molecules secreted by the fibroblasts may exert regulatory effects on cardiomyocytes (2,4,5) or cardiac vessels (6,7).

Cardiac fibroblasts are involved in the regulation of the heart extracellular matrix under both physiological and pathological conditions (8). Collagen, the fibrotic protein of the extracellular matrix, not only provides mechanical support for cardiomyocytes (9), but is also responsible for the distribution of mechanical force within the heart (2) and can influence the electrophysiological processes within the myocardium (10). Collagen content determines compliance of the heart. Cardiac fibroblasts can be transformed into a profibrotic phenotype, known as myofibroblasts (11), which exhibit elevated migratory and proliferative capacity, and secrete a range of bioactive molecules (12).

The human heart is also home to mast cells (13). These can secrete proinflammatory, angiogenic and lymphangiogenic factors, and may participate in the pathogenesis of heart disease (13). They are also an important source of histamine, and histidine decarboxylase, the enzyme responsible for histamine synthesis, has been found within the heart (14). Histamine was found to exert positive chronotropic and inotropic effects on the hearts of rats overexpressing the H_2 receptors, and these effects were observed *in vivo* and *in vitro* (15). After myocardial infarction, the number of mast cells increases in the myocardium (16), and histamine concentration within the blood is elevated (17). Moreover, histamine may promote

fibrosis within the transgenic mice via the H_2 receptors (17). Although histamine regulates fibrosis in the granulation tissue of the wound (18), it was not found to influence the fibroblasts of the intact skin (18). In addition, rat myofibroblasts from the myocardial scar were found to be subject to the regulatory action of histamine (19).

The aim of the present study was to determine whether histamine exerted a regulatory influence on collagen accumulation in cells derived from intact heart tissues, and to examine the participation of histamine in heart fibrosis initiation. Additionally, the histamine receptor involved in heart fibrosis regulation was identified, as well as the types of the isolated cells.

Materials and methods

Cell culture and experimental design. The cells (stored in liquid nitrogen) were obtained from control rats of another project, which was approved by the Local Commission of Ethics in Łódź (Łódź, Poland; approval no. 46ŁB 624/2012).

Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with FBS (Biowest), gentamycin (25 μg/ml) amphotericin (2.5 μg/ml) at 37˚C in a humidified incubator, supplied with 95% air and 5% CO_2. Defrosted cells were plated at an initial cell density of $8x10^4$ cells per well in a 6-well plate. The number of cells were counted using trypan blue staining at room temperature (5 min) in a Bürker chamber. After cells had reached confluence (70%), the cells were trypsinized, passaged in new flasks and used for subsequent experiments.

In vitro experiments. Myofibroblasts were grown in DMEM supplemented with 3% FBS and antibiotics as described above. The first part of the study investigated the effects of histamine (Sigma-Aldrich; Merck KGaA) on collagen content in the myofibroblast culture. Histamine was used at concentrations ranging from $1x10^{-10}$-$1x10^{-5}$ M. The results were compared with control (untreated cells).

In the second portion of the study, receptor agonists were used to identify receptors involved in the histamine-dependent effects: 2-pyridylethylamine dihydrochloride (H_1 receptor agonist) (Tocris Bioscience), amthamine dihydrobromide (H_2 receptor agonist) (Tocris Bioscience), imetit (H_3 receptor agonist; Sigma-Aldrich; Merck KGaA), 4-methylhistamine hydrochloride (H_4 receptor agonist; Sigma-Aldrich; Merck KGaA). The agonists were applied at concentrations of 1x10-8, $1x10^{-6}$ and $1x10^{-4}$ M, respectively.

The third portion of the study examined the effects of the histamine receptor inhibitors ketotifen (H_1-receptor inhibitor; Sigma-Aldrich; Merck KGaA) and ranitidine (H_2-receptor inhibitor; Sigma-Aldrich; Merck KGaA). The cells were divided into four groups: Untreated controls, cells treated with histamine ($1x10^{-6}$ M), a group treated with both histamine ($1x10^{-6}$ M) and histamine receptor inhibitor ($1x10^{-5}$ M), and a group treated only the with histamine receptor inhibitor ($1x10^{-5}$ M).

The inhibitors of the H_3 (ciproxifan; $1x10^{-5}$ M) and H_4 (JNJ7777120; $1x10^{-5}$ M; Sigma-Aldrich; Merck KGaA) receptors were investigated. These experiment were comprised of the following groups: Controls, cells treated with 0.001% DMSO (antagonist solvent); cells treated with $1x10^{-6}$ M histamine; cells treated with ciproxifan alone; cells treated with JNJ7777120 alone; cells treated with $1x10^{-6}$ M histamine and ciproxifan; and cells treated with $1x10^{-6}$ M histamine and JNJ7777120). All groups in this portion of the study, except the untreated control, were treated with 0.001% DMSO. Each group consisted of 8 or 9 repeats.

Reverse transcription-quantitative (RT-q)PCR. Gene expression was measured in four separate cultures from each group, and each sample was measured twice. Total RNA from cells was extracted using a Total RNA Mini kit (A&A Biotechnology). Reverse transcription and cDNA synthesis was performed using a PrimeScript RT-PCR kit according to the manufacturer's protocol (Takara Bio, Inc.).

The expression of the collagen type I and III genes was measured, and GAPDH, hypoxanthine-guanine phosphoribosyltransferase (hprt1) and 60S ribosomal protein L13a (rpl13a) were used as the reference genes. The genes coding for the α1 chain of procollagen type I, procollagen type III and hprt1, rpl13a were evaluated using an Universal Probe Library (UPL; Roche Diagnostics GmbH). Real Time ready Custom Single assays (Roche Diagnostics) were used to measure GAPDH expression. The sequences of the primers used were: Collagen type 1 forward, GGGATTCCCTGGACCTAAAG and reverse, GGAACACCTCGCTCTCCA, UPL probe #67; collagen type III forward, TCCCCTGGAATCTGTGAATC and reverse TGAGTCGAATTGGGGAGAAT, UPL probe #49; rpl13a forward, CCCTCCACCCTATGACAAGA and reverse, GGTACTTCCACCCGACCTC, UPL probe #74; and hprt1 forward, CTCCTCAGACCGCTTTTCC and reverse, TCATAACCTGGTTCATCATCA, UPL probe #95.

The reactions were performed using the Fast Start Essential Probe MasterMix (Roche Diagnostics GmbH) with the following thermocycling conditions: Initial incubation at 95˚C for 10 min; followed by 55 cycles of 95˚C for 10 sec, 60˚C for 30 sec, and incubation at 72˚C for 1 sec; with a final incubation at 40˚C for 30 sec. A LightCycler® 96 software (Roche Diagnostics) was used to calculate the relative gene expression (20).

Flow cytometry. The expression of α-smooth muscle actin, vimentin and desmin within cells was assessed using flow cytometry. In the first step, the cells were fixed at 4˚C for 30 min (Fixation Buffer; BD Biosciences) and then permeabilized with Perm Buffer (BD Biosciences). In the next step, the cells were stained at a temperature of 6˚C for 30 min in Stain Buffer (BD Biosciences). Subsequently, the tested cells were treated with specific antibodies conjugated to FITC at 4˚C for 30 min. For each experiment, ~10,000 cells were assessed on the flow cytometer. The following antibodies were used for the experiment: α-smooth muscle actin Antibody (cat. no. NBP-2-34522F; 1:500; Novus Biologicals); mouse IgG2a κ-Light Chain Isotype control (cat. no. NBP1-43955; 1:500; Novus Biologicals); Desmin antibody (cat. no. DES/1711; 1:500; Novus Biologicals); mouse IgG1 κ-Light Chain Isotype control (1:500; Novus Biologicals); Vimentin Antibody (cat. no. LN-6; 1:250: Novus Biologicals); and mouse IgM Isotype control (1:250; eBioscience, Inc; Thermo Fisher Scientific, Inc.). A FACS Canto II Analytical Flow

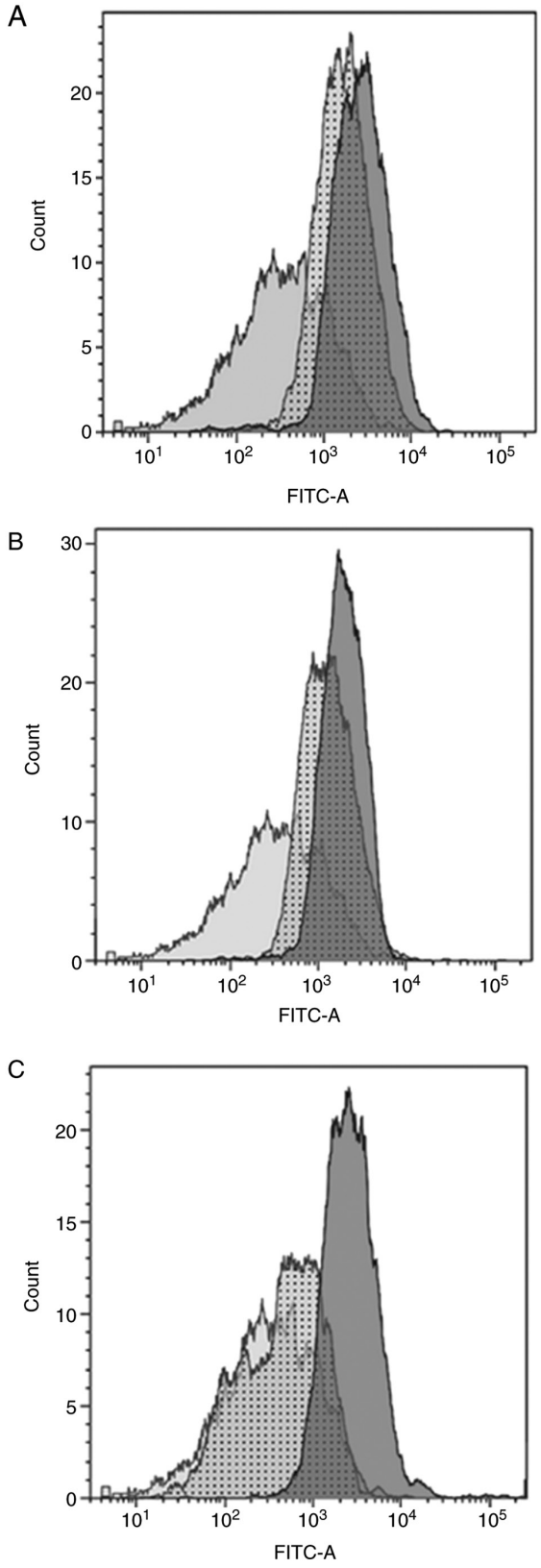

Figure 1. Flow cytometry analysis to determine the types of cells. FACS profiles indicate the expression of (A) α-smooth muscle actin, (B) desmin and (C) vimentin in myofibroblasts (right curve), isotypic control (middle curve) and untreated control cells (left curve).

Cytometer (BD Biosciences) was used for analysis. FACS data were analyzed using Kaluza Analysis version 1.5a (Beckman Coulter, Inc.).

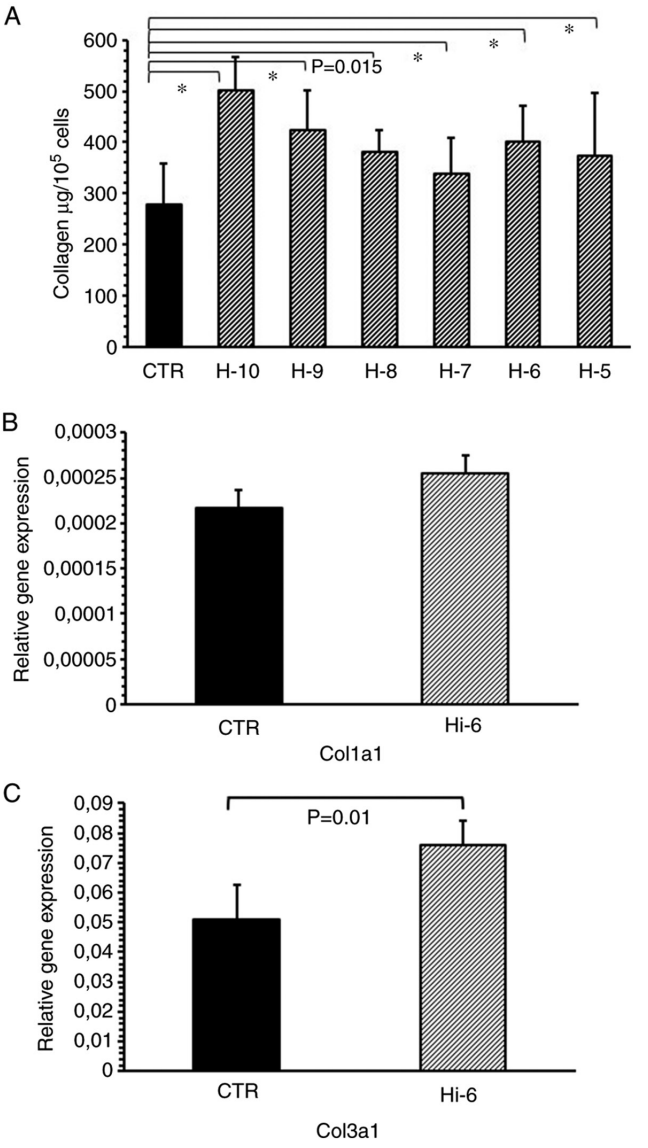

Figure 2. Total collagen content in isolated heart cells treated with histamine. (A) Collagen content in cells isolated from hearts in the CTR or cells treated with different concentrations of histamine. *P<0.001 vs. control. Relative gene expression of (B) Col1a1 and (C) Col3a1 in the myofibroblasts cultures isolated from the heart treated with histamine. Data are presented as the mean ± standard deviation. CTR, control; Col1a1, α1 chain of procollagen type I; Col3a1, α1 chain of procollagen type III; H-10, $1x10^{-10}$ M; H-9, $1x10^{-9}$ M; H-8, $1x10^{-8}$ M; H-7, $1x10^{-7}$ M; H-6, $1x10^{-6}$ M; H-5, $1x10^{-5}$ M.

Determination of collagen levels. The total collagen levels in the cultured cells were evaluated using the Woessner method (14). The samples were dried at 60˚C and then hydrolyzed with 6 N HCl (3 ml/10 mg dry tissue) at 100˚C for 24 h in a water bath. The hydrolysates were evaporated and the precipitates were dissolved in 3 ml deionized water. Subsequently, the samples were neutralized with 1 N NaOH and diluted to 10 ml with deionized water. For analysis, 0.2 ml sample was taken and diluted with redistilled water to a final volume of 2 ml. The sample was suspended in 1.25 ml Chloramine T in citrate buffer (pH 6.0), shaken for 5 min and incubated at room temperature for 20 min. During this time, the pyrrole was oxidized to hydroxyproline by chloramine T. To remove the excess chloramine T, 1 ml perchloric acid (3.15 M) was added. After 5 min, the samples were treated

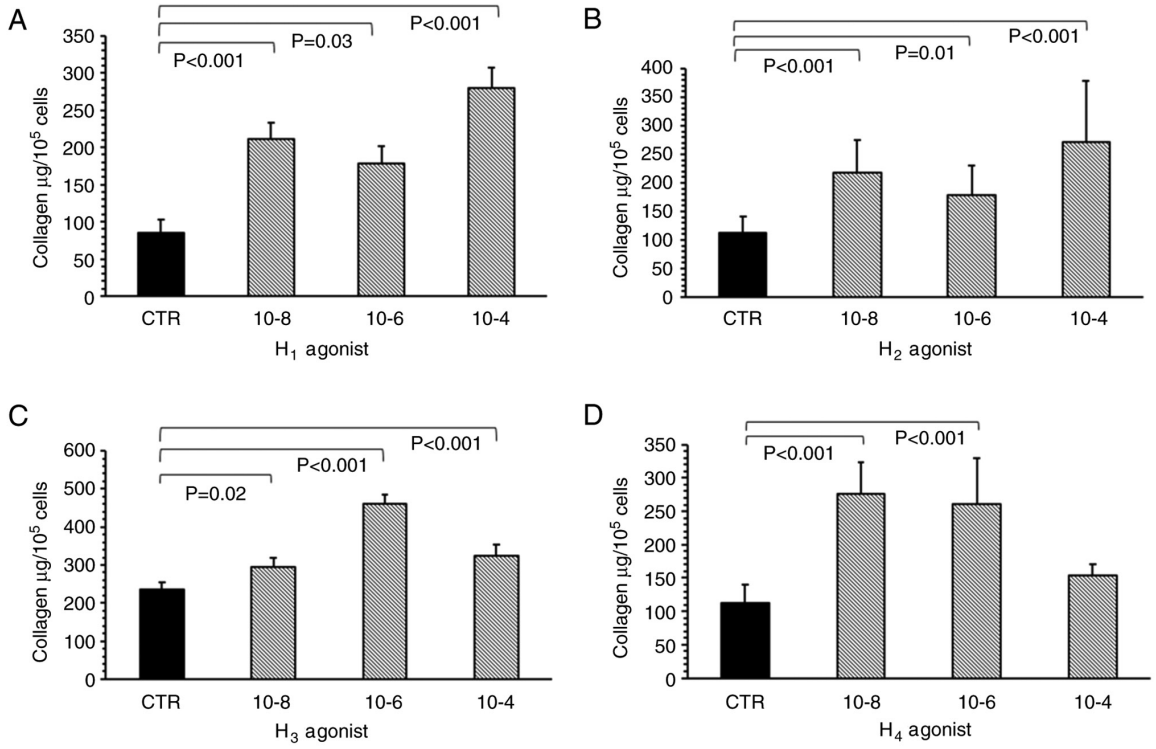

Figure 3. Total collagen content in isolated heart cells treated with histamine receptor agonists. Collagen content in cells isolated from hearts in the CTR or cells treated with different concentrations of the (A) H_1 receptor agonist (2-pyridylethylamine dihydrochloride), (B) H_2 receptor agonist (amthamine dihydrobromide), (C) H_3 receptor agonist (imetit), or (D) H_4 receptor agonist (4-methylhistamine hydrochloride). Data are presented as the mean ± standard deviation. CTR, control; 10-8, $1x10^{-8}$ M; 10-6, 10^{-6} M; 10-4, 10^{-4} M.

with 1 ml 20% p-dimethylaminobenzaldehyde and incubated in a water bath at 60˚C for 20 min. The optical density of the samples was scanned at 560 nm on a spectrophotometer.

Statistical analysis. Data were compared using a Kruskal-Wallis test. Differences between all groups were compared using a multiple comparisons of mean ranks test (Dunn's test). P<0.05 was considered to indicate a statistically significant difference. Statistica version 13 (StatSoft, Inc.) was used for statistical analysis.

Results

Flow cytometry. The isolated cells were found to have longitudinal, fusiform, spindle and stellate morphology. The flow cytometry experiments showed that the structure of isolated cells was typical for myofibroblasts (20-22); they were positive for all tested markers: α-smooth muscle actin (Fig. 1A), desmin (Fig. 1B) and vimentin (Fig. 1C). The expression of these markers indicates that this cell culture could be distinguished from smooth muscle cells (vimentin negative, and positive for both α-smooth muscle actin and desmin) and fibroblasts (negative for α-smooth muscle actin, and positive for both desmin and vimentin) (23).

RT-qPCR. Histamine at concentrations ranging from $1x10^{-10}$-$1x10^{-5}$ M significantly increased the collagen levels in the cultured myofibroblasts compared with the untreated control (Fig. 2A). The maximal effect was observed for histamine administered at $1x10^{-10}$ M (P<0.001) and $1x10^{-9}$ M (P<0.001). Histamine ($1x10^{-6}$ M) increased the expression of the collagen type III gene. (Fig. 2B and C).

In vitro experiments. All histamine receptor agonists, used to mimic the effects of histamine, significantly increased collagen levels in the myofibroblast culture. The H_1 receptor agonist (2-pyridylethylamine dihydrochloride) significantly increased collagen deposition compared with the control, at all tested concentrations ($1x10^{-8}$ M, P<0.001; $1x10^{-6}$ M, P=0.03; and $1x10^{-4}$ M, P<0.001; Fig. 3A). The most potent effect was observed when cells were treated with $1x10^{-4}$ M of the H_1 receptor agonist. The H_2 receptor agonist (amthamine dihydrobromide) had a statistically significant effect on collagen level in the myofibroblast cultures at all tested concentrations (Fig. 3B). Specifically, $1x10^{-8}$ (P<0.001), $1x10^{-6}$ (P<0.01) and $1x10^{-4}$ M (P<0.001) H_2 receptor agonist significantly increased collagen content within the cell cultures compared with the control.

Imetit, the H_3 receptor agonist, increased the collagen content compared with controls when administered at $1x10^{-8}$ (P=0.02), $1x10^{-6}$ (P<0.001) and $1x10^{-4}$ M (P<0.001) (Fig. 3C), with the maximum effect observed at $1x10^{-6}$ M. Elevated collagen levels were also observed for $1x10^{-8}$ (P<0.001) and $1x10^{-6}$ M (P<0.001) 4-methylhistamine hydrochloride, the H_4 receptor agonist (Fig. 3D); however, no such increase was observed at $1x10^{-4}$ M.

Histamine significantly increased collagen levels in the myofibroblast cultures compared with controls at a concentration of $1x10^{-6}$ M (P=0.03; Fig. 4A). Treatment with $1x10^{-5}$ M ketotifen, an H_1 receptor inhibitor, did not modify the effects

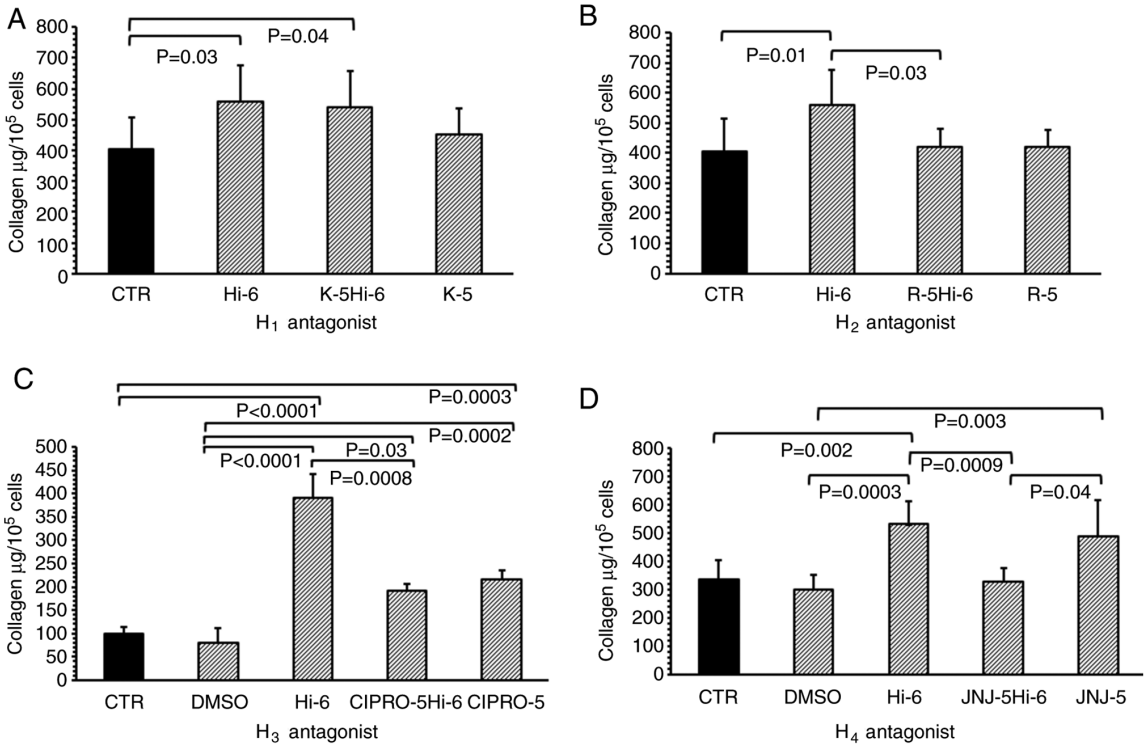

Figure 4. Total collagen content in isolated heart cells treated with histamine receptor antagonists. (A) Collagen content in myofibroblasts culture isolated from the heart in the CTR or cells treated with Hi-6, K-5 (H_1 receptor antagonist) or both (K-5Hi-6); (B) treated with Hi-6, R-5 (H_2 receptor antagonist) or both R-5Hi-6; (C) treated with DMSO, Hi-6, CIPRO-5 (H_3 receptor antagonist) or both (CIPRO-5Hi-6); and (D) DMSO, Hi-6, J-5 (H_4 receptor antagonist) or both (J-5Hi-6). Data are presented as the mean ± standard deviation. CTR, control; Hi-6, $1x10^{-6}$ M histamine; K-5, $1x10^{-5}$ M ketotifen; R-5, $1x10^{-5}$ M ranitidine alone; DMSO, 0.001% DMSO; CIPRO-5, $1x10^{-5}$ M ciproxifan; J-5, $1x10^{-5}$ M JNJ7777.

of histamine when compared with histamine alone; the levels of collagen in the ketotifen and histamine-treated groups were still higher than that in the controls (P=0.04). Ketotifen (without histamine), applied to the cultures of myofibroblasts at a concentration of $1x10^{-5}$ M, did not alter the collagen content in these cultures when compared with the control (Fig. 4A).

The H_2 receptor inhibitor Ranitidine ($1x10^{-5}$ M) significantly reduced the effects of histamine ($1x10^{-6}$ M) when compared with cells treated with histamine alone (P=0.03; Fig. 4B). Ranitidine (without histamine), applied at a concentration of $1x\ 10^{-5}$ M to the cell cultures, did not change the collagen content when compared with the control group.

The control and DMSO-treated cultures were found to have similar collagen levels. In addition, histamine increased the levels of collagen within the culture when compared with the untreated controls (P<0.001) and DMSO-treated (P<0.001) cells. Ciproxifan, the H_3 receptor inhibitor, applied at concentrations of $1x\ 10^{-5}$ M, blocked the effects of histamine, decreasing the levels of collagen when compared with histamine alone (P=0.0008). Furthermore, the groups treated with ciproxifan and histamine, or ciproxifan alone, demonstrated augmentation of collagen content when compared with the untreated controls (P=0.0003) or cells treated with DMSO (P=0.0002; Fig. 4C).

Comparable levels of collagen were found in the untreated controls and DMSO-treated cultures; however, higher collagen levels were observed in fibroblast cultures treated with histamine when compared with the control (P=0.002) and DMSO (P=0.0003) treated groups. JNJ (H_4 receptor inhibitor) blocked histamine-induced collagen elevation in the culture (P=0.0009; Fig. 4D) when compared with cells treated with histamine alone. Higher levels of collagen were found in the JNJ-treated fibroblasts compared with the DMSO treated group.

Discussion

In addition, flow cytometry analysis showed that the cells were α-smooth muscle actin positive, vimentin positive and desmin positive. These features differentiate them from smooth muscle cells, which are desmin and α-smooth muscle actin positive, but vimentin negative, and fibroblasts, which are negative for α-smooth muscle actin, but positive for both desmin and vimentin (23,24). The cells isolated in the present study from intact hearts are hypothesized to transform into myofibroblasts during isolation or culture. Recent data has indicated that several factors may be responsible for this transformation, and it has been suggested that it may be induced by increased substrate stiffness amongst the cultured cells (25). An intrinsic mechanotransduction mechanism may also be involved in the transformation of cells into myofibroblasts (26).

Histamine appears to have a significant influence on collagen accumulation in the heart myofibroblast cultures at all applied concentrations. This observation clearly suggests that histamine augments collagen content by acting directly on the myofibroblasts, and that unstimulated myofibroblasts from the intact heart may respond to histamine by increasing collagen content within the cultures. These data correspond with our previous studies, suggesting that histamine may

increase the collagen level within cultures of myofibroblasts derived from the heart myocardial infarction scar (19), and that it may also accelerate the metabolism of the myofibroblasts and increase collagen content within the myofibroblasts derived from granulation tissue of skin wound model of the rats (18). This effect corresponds with increased secretion of TGF-β1 following histamine treatment. Indeed, previous studies performed on myofibroblasts derived from wounds or myocardial infarction indicate that cells subjected previously to pro-inflammatory factors may respond to histamine (19,18). However, amongst cultures of fibroblasts derived from intact skin, treatment with high ($1x10^{-4}$-$1x10^{-6}$ M) or low ($1x10^{-9}$ M) concentrations of histamine augment collagen type I content (27). These effects were confirmed by Takeda *et al* (28) on human foreskin fibroblasts. Histamine exerts its effect on collagen content via stimulation of procollagen type III expression; however, no such effect is observed with regard to procollagen type I.

The tested inhibitors of the H_2, H_3 and H_4 histamine receptors were found to block histamine-induced elevation of collagen content. These findings are supported by the observation that the H_2, H_3 and H_4 agonists mimicked histamine action and augmented collagen content within the cultures. Such expression of H_2, H_3 and H_4 histamine receptors, and the confirmation of their activity within the heart has been reported previously (29-31). Interestingly, the H_2-receptor agonist amthamine dihydrobromide demonstrated its maximal effect at a high relatively concentration ($1x10^{-4}$ M), whereas imetit activity (an H_3 receptor agonist) peaked at $1x10^{-6}$ M, and the H_4-receptor agonist 4-methylhistamine hydrochloride demonstrated maximal activity at both $1x10^{-6}$ and $1x10^{-8}$ M. Hence, stimulation of the H_2, H_3 and H_4 histamine receptors may be responsible for the collagen augmentation observed within the cardiac myofibroblast cultures.

Neither histamine itself nor any of the receptor agonists tested in the present study were found to exert a concentration-dependent effect, consistent with our previous study (19). This effect has been attributed to saturation of the receptors by lower concentrations of the tested compounds. In contrast, the results of the present study suggest that the three histamine receptors act in accord, and the blockade of one receptor by a single antagonist negates the full effect of histamine; however, this hypothesis required further investigation. However, different effects were observed for receptor H_1. The H_1-receptor agonist 2-pyridylethylamine dihydrochloride increased collagen content but the H_1 receptor antagonist ketotifen did not block histamine action. Therefore, the present study failed to confirm whether the H_1 receptor regulates collagen content in cardiac myofibroblasts. The effect of 2-pyridylethylamine dihydrochloride is hypothesized to be unspecific; it is not dependent on the H_1 receptor.

Previously, it has been proposed that in the cardiac myofibroblasts obtained from a myocardial infarction scar, the histamine content is mediated by the H_3 receptor (19). However, the effect of histamine was found to be influenced by the H_1 receptor in myofibroblasts from wound granulation tissue (18,32). H_1 receptor activation appears to be involved in the regulation of collagen metabolism within dermal fibroblasts (27), whereas blockade of the H_2 receptor decreased collagen type I gene expression in human fibroblasts (28). These results were also observed *in vivo*: H_2 histamine receptors appear to be involved in the regulation of collagen levels in a model of cutaneous wounds in rats (33), and both H_1 and H_2 receptors may regulate collagen levels in fibroblast-like cells (34). H_3 histamine receptors may also regulate collagen content within myofibroblasts derived from a myocardial infarction scar (19). Hence, it appears that different histamine receptors may be involved in the regulation of collagen content, although the obtained results are dependent on the experimental model, and the selected tissue or species. Previous studies have suggested that different types of histamine receptors participate in the regulation of collagen deposition in various organs or tissues (19,32-34). This phenomenon could be dependent on the varying influence of the extracellular environment on myofibroblasts (2). The results of the present study indicate that in myofibroblasts derived from intact rat heart, the H_2, H_3 and H_4 histamine receptors participate in regulation of collagen accumulation within the culture. In contrast, in myofibroblasts taken from a myocardial infarction scar, only the H_3 histamine receptor was previously confirmed to influence collagen deposition (19). The myocardial scar myofibroblasts were subjected to stimulation by mediators released by the inflammatory environment (35). The activity of some types of histamine receptors participating in fibrosis regulation is hypothesized to be dependent on mediators released during healing or inflammatory processes.

Histamine is hypothesized to act on collagen content via a number of autocrine or paracrine effects (18,36). Histamine stimulates the release of profibrotic TGF-β1 by myofibroblasts from granulation tissue of a rat wound model in an H_1-dependent manner (18). Moreover, histamine is known to increase secretion of ATP via activation of pannexin-1 hemichannels and to increase collagen type I content within the subcutaneous fibroblast cultures (36).

The results of the present study indicate that histamine may directly increase collagen content on rat heart myofibroblasts, possibly by augmentation of procollagen type III expression. This effect is dependent on H_2, H_3 and H_4 histamine receptor stimulation. These results highlight the role of histamine in heart fibrosis and suggest that histamine receptors may be potential targets for antifibrotic therapy.

Acknowledgements

We would like to thank Mrs Teresa Staszewska (Department of Behavioral Pathophysiology, Medical University of Łódź, Łódź, Poland) for her excellent technical assistance.

Funding

The present study was supported by a grant from the Medical University of Łódź (grant no. 503/6-103-04/503-01).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LP and MJ participated in the study design and analysis of the data, performed all the experiments and participated in the manuscript preparation. JS assisted with data analysis and interpretation. JD supervised the laboratory analyses, assisted with the design of the study and participated in manuscript preparation. All authors have read and approved the final manuscript. LP, JS, MJ and JD confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The cells (stored in liquid nitrogen) were obtained from control rats of another project, which was approved by the Local Commission of Ethics in Łódź (approval no. 46ŁB 624/2012).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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