

Possible contribution of histamine to the physiopathology of hepatic fibrosis: modulation of proteolytic activity

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ABSTRACT

This study was carried out to determine whether histamine modulates the production, activity and secretion of urokinase-like plasminogen activator and type IV collagenase in the hepatic cell line WRL-68, in order to infer on the molecular mechanisms that might explain the role of histamine in hepatic fibrogenesis. Our results show that histamine increases the activity of both proteases. The activity of uPA was doubled at all tested concentrations of histamine, whereas type IV collagenase was increased only with 75 μ M histamine ($p < 0.05$). Although the physical amounts of collagenase IV and uPA proteins were markedly increased in both the cells and conditioned medium, the levels of proteolytic activity were not increased in the latter. The present results indicate that histamine induces the intracellular production of functionally active proteases in WRL-68 cells, and that even though it also increases the secretion of these proteases to the conditioned medium, the secreted proteins are not active. Our data also suggest that histamine mediates its effects on liver cells through the H1 receptor pathway, because only pyrilamine inhibited the effects of the histamine treatment. Further studies are necessary to understand the role of histamine in fibrogenesis.

Key words: uPA, collagenase IV, proteases, fibrosis

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RESUMEN

Posible contribución de la histamina en la fisiopatología de la fibrosis hepática: modulación de la actividad proteolítica. La finalidad de este estudio fue determinar si la histamina regula la producción, actividad y secreción del activador de plasminógeno tipo urocinasa (uPA) y colagenasa IV en células hepáticas WRL-68 y con esto inferir mecanismos moleculares que expliquen el rol de la histamina en la fibrogénesis hepática. Nuestros resultados muestran que la histamina induce la actividad de ambas proteasas. Se observó que en las células WRL-68 se incrementó la actividad de uPA (2 veces) con todas las concentraciones de histamina, mientras que la actividad de la colagenasa IV solo se elevó con 75 μ M de histamina ($p < 0.05$). La actividad de las proteasas no se incrementó en el medio condicionado. En contraste los niveles de las proteínas colagenasa IV y uPA fueron marcadamente incrementados tanto en células como en el medio condicionado. Estos resultados indican que la histamina induce la producción de proteasas funcionalmente activa en células hepáticas WRL-68. Histamina es capaz de inducir la secreción de las proteasas al medio condicionado, pero esas proteínas no son activas. Nuestros resultados sugieren que la histamina media sus efectos a través del receptor H1, debido a que solamente la pirilamina fue capaz de abrogar el efecto anteriormente referido. Estudios posteriores son necesarios para comprender el papel de la histamina en la fibrogénesis.

Palabras clave: uPA, colagenasa IV, proteasas, fibrosis

Introduction

Hepatic fibrosis is the result of an alteration in the balance between protein synthesis, degradation and deposition at the extracellular matrix. The resulting changes in composition of the matrix are similar for all forms of chronic hepatic damage, suggesting the same mechanism for alcoholic, viral and biliary fibrosis [1-3]. At the cellular level, fibrogenesis is triggered by a damage to hepatocytes that leads to the recruitment of inflammatory cells and platelets, as well as to the activation of Kupffer and stellate cells with a consequent release of cytokines and growth factors [4, 5]. Stellate cells are the main target of the inflammatory stimulus and play a central role in this process due to their capacity for activation after liver damage. This activation is a response defined by morphological and functional changes related to their proliferation, contractility, fibrogenesis, cytokine

secretion and degradation of the extracellular matrix. [6, 7]. The elements controlling their activation are complex and multifactorial, but it is widely agreed that cytokines and growth factors are among the most important mediators responsible for stellate cell activation both *in vitro* and *in vivo* [8-10].

Histamine is a ubiquitous chemical mediator found in body fluids and tissues that can also function as a growth factor [11]. The interesting observation that histamine synthesis is induced in tissues undergoing rapid proliferation or recovering from cellular damage suggests that it has other still unknown roles [12]. So far, 4 subtypes of histamine receptors have been found in humans (H1, H2, H3, H4), all belonging to the family of G protein-coupled receptors [13-15]. It has been reported that histamine receptors are expressed in multiple types of both normal and tumor cells,

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where they can be activated by several different signaling pathways, thus triggering a diverse array of biological effects [16, 17].

Hepatic fibrosis constitutes a complex physiopathological reaction that involves all resident liver cells through several autocrine, paracrine and juxtacrine signal relay mechanisms [18-22]. It is a consequence of changes not only in the secretion, but also in the degradation of the extracellular matrix, which means that in chronic hepatic disease the functional dynamic balance between fibrogenesis and fibrolysis has been lost. Although it has been reported that histamine is not a cause of fibrosis [23], other evidence, including ours, have showed the involvement of histamine in the physiology of liver cells and in the synthesis of factors responsible for acute and chronic liver damage [24-26]. Therefore, the possible contribution of histamine to the physiopathology of hepatic fibrosis can not be dismissed.

The urokinase plasminogen activator (uPA) is a protease at the top of a proteolytic cascade involved in remodeling the extracellular matrix on both physiological and pathological conditions [27, 28]. It has been shown that uPA plays an important role in the physiopathology of chronic hepatic disease. Several *in vitro* models have demonstrated the presence and activity of collagenases and uPA on stellate cells and hepatocytes during the development of hepatic fibrosis [29-31]. Since some published reports indicate that the levels of hepatic and serum histamine increase during acute or chronic hepatic damage, it is not possible to discard the possibility of a protease-histamine interaction on the physiopathology of hepatic disease. Therefore, this study is aimed at determining whether histamine regulates the production, activity and secretion of the urokinase plasminogen activator and type IV collagenase in WRL-68 hepatic cells.

Materials and methods

Cell lines and culture conditions

WRL-68 is a cell line derived from normal human hepatic tissue (ATCC, CL-48). For this study, the cells were seeded and maintained in Minimal Essential Medium (MEM, Gibco BRL), 4 mM glutamine, 1% essential aminoacids (Gibco BRL), 10% fetal calf serum and ampicillin at 100 µg/mL. A total of 10⁶ cells was seeded in a 75 cm² culture flask and maintained for 24 hours at 37 °C under a 5% CO₂ atmosphere and humidity. Subculturing was done with a trypsin-EDTA (N,N,-di-ethylthiocarbamic acid sodium) solution.

Pharmacological treatments

For the different experimental treatments, 4 x 10⁶ cells were seeded on 100 mm culture plates at 50% confluence and cultured for 24 hours. At the end of this period the medium was discarded and the cells were washed twice with PBS, after which they were incubated on MEM containing transferrin at 10 µg/mL, L-glutamine, non-essential aminoacids and antibiotics, together with the treatment substance. The following treatment groups were included: (a) Control (no treatment); (b) Histamine (10, 50, 75 and 100 µM); (c) Pylramine (1 µM); (d) Cimetidine (1 mM); (e)

Histamine (75 µM) + Pylramine (1 µM), and (f) Histamine (75 µM) + Cimetidine (1 mM). The pylramine and cimetidine groups (H₁ and H₂ antagonists, respectively) were included to identify the receptor type involved, and their concentrations (1 µM for pylramine, 1 mM for cimetidine) were chosen to be comparable with those used in previous studies. After a further 24 h culture period, both the cells and the conditioned medium were collected and stored frozen at -20°C until used.

Analysis of proteolytic activity

A zymographic analysis was performed on polyacrylamide-sodium dodecyl sulphate gels (SDS-PAGE) to quantify the activity of uPA and type IV collagenase, as previously described by Miskin and Soreq [31]. Briefly, culture and cell extract samples (20 µg of total protein) were subjected to electrophoresis on polyacrylamide-sodium dodecyl sulphate gels containing plasminogen or gelatin as substrates for uPA and type IV collagenase, respectively. After the electrophoretic run, the gels were washed in 2.5% Triton X-100 to remove the sodium dodecyl sulphate, and incubated for 3 hours at 37 °C to allow proteolysis to proceed. Afterwards, the gels were stained with Coomassie Blue (R-250; BioRad). The activity of uPA and type IV collagenase was monitored through the appearance of clear bands in which the plasminogen or gelatin embedded on the gel has been degraded. In all cases, the clear bands showed the expected electrophoretic mobility of the proteins under study, as assessed by comparison to molecular weight standards. No bands were detected in the control gel runs without plasminogen or gelatin. For a semiquantitative examination of the results, the gel images were digitized and analyzed by densitometry, quantifying the differences between the bands with an image analyzer (Quantity One, BioRad).

Immunoassays

The total amount of type IV collagenase and uPA was assayed through immunoenzymatic tests using polyclonal antibodies against these proteins (ELISA-direct enzyme-linked immunosorbent assay) [32]. These antibodies were obtained by immunizing New Zealand rabbits. Both the cell extracts and the conditioned culture media from each treatment were quantified. The assays were performed on 96-well immunoassay plates by the direct competition method to detect soluble antigens. Briefly, 0.2 µg of antigen diluted in carbonate buffer were bound to the plates by incubation for 2 h at 37 °C, after which non-specific binding sites were blocked by overnight incubation at 4 °C with bovine serum albumin diluted in PBS. After blocking, 100 µg of total protein from each of the assayed samples were mixed with the anti-type IV collagenase or anti-uPA sera. A standard competition curve was prepared for each assay, containing serial dilutions of the antigen in the blocking solution previously mixed with its antibody (serum) and incubated for 30 minutes at room temperature. Once the samples and the curve were ready, they were dispensed into the wells containing the bound antigen and incubated at 37 °C for 2 h. Finally, a chromogenic substrate was added and the reaction was stopped after 10 minutes

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by adding 6 N HCl. Optical density was measured on a plate reader (Dynatech Micro ELISA Reader), at a wavelength of 492 nm.

Statistical analysis

All the values are expressed as a mean \pm SEM of the readings obtained from 5 duplicated experiments. The statistical analysis was conducted using a Fisher test, and values of P lower than 0.05 were regarded as significant.

Results

Type IV collagenase activity on hepatic cells and conditioned media.

A proteolytic activity mediated by type IV collagenase was detected through the appearance of 72 kDa degradation bands on the zymograms (Figure 1a). This activity was detected only in cells treated with 75 μ M histamine (2.5-fold higher than the control), $p < 0.05$ (Figure 2). Pyrilamine and cimetidine groups (H1 and H2 antagonists, respectively) were included to identify the type of histamine receptor involved, as mentioned previously. The collagenase proteolytic activity was 100% inhibited on cells simultaneously treated with histamine (75 μ M) and pyrilamine (1 μ M) $p < 0.05$; that is, pyrilamine completely inhibited the effect of histamine, whereas no changes in proteolytic activity were noted on cells simultaneously treated with histamine and cimetidine.

The pattern of collagenase proteolytic activity on the conditioned media closely follows that of the cell extracts, that is, treatment with 75 μ M histamine increases the activity significantly (4-fold higher than the control), and to a lower extent, so does the treatment with 100 μ M histamine, $p < 0.05$ (Figure 2). Analyzing the effect of the antagonists, it can be noted that there was 100% inhibition of the proteolytic activity on the conditioned media from cells treated with 1 μ M pyrilamine plus 75 μ M histamine, ($p < 0.05$), but not upon addition of cimetidine.

Activity of uPA on hepatic cells and conditioned media

A proteolytic activity mediated by uPA was detected in all the treatment groups when monitoring

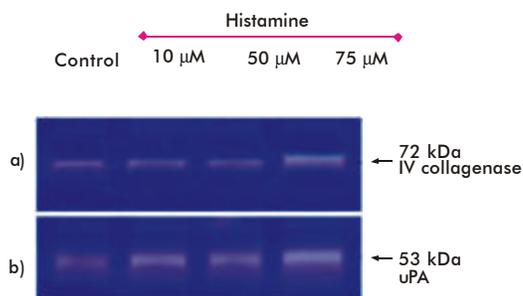


Figure 1. Effect of histamine on the proteolytic activity of type IV collagenase and uPA, in WRL-68 cells. Type IV collagenase- and uPA-mediated proteolytic activity was detected by the presence of clear bands in the zymographic assays from cell extracts (20 μ g of protein). (a) Type IV collagenase activity on gelatin gels, MW of 72 kDa. (b) Activity of uPA on plasminogen gels, MW of 53 kDa.

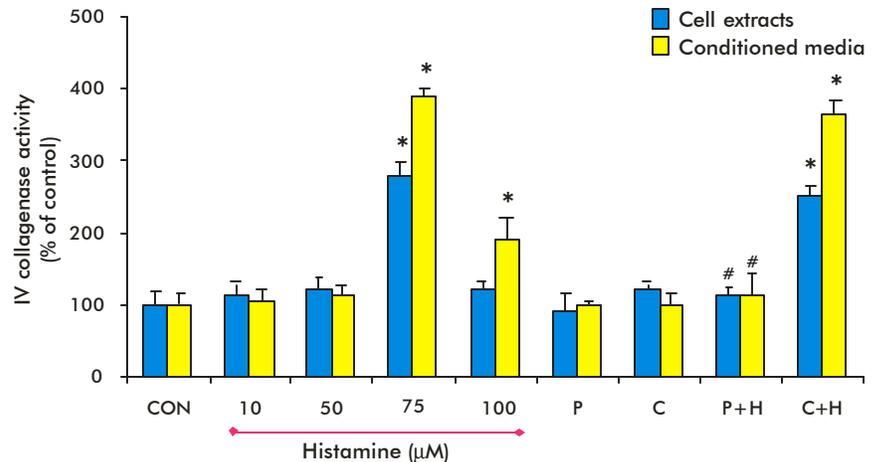


Figure 2. Effect of histamine treatment on type IV collagenase activity. Cells were treated for 24 h. with different histamine concentrations. Pyrilamine (P) at 1 μ M or Cimetidine (C) at 1 mM, which are H1 and H2 antagonists respectively, were added simultaneously with 75 μ M histamine (H) or alone. Protein samples containing 20 μ g were electrophoresed and analyzed by zymography. The density of the bands was quantified with imaging software, and the results were expressed as a percentage relative to the control. Bars with different symbols are significantly different from the control group (* $p < 0.05$) or from the group treated with 75 μ M histamine (# $p < 0.05$).

the appearance of bands on the zymograms. This proteolytic activity proved to be dependent on plasminogen by subjecting the same samples to runs on gels without plasminogen. The presence of an active 53 kDa band related to uPA is consistent with previous reports in the literature [22]. Our results showed the presence of uPA-mediated proteolytic activity on cell extracts from every tested histamine concentration, at levels much higher than those found for collagenase (Figure 1b). The activity was increased more than 100% when compared to the control cells, $p < 0.05$ (Figure 3). Pyrilamine completely inhibited (100%) the effect of histamine on uPA activity, $p < 0.05$, whereas 1 mM cimetidine was ineffective. Both pyrilamine and cimetidine tend to increase uPA activity in cell extracts, but this increase was not statistically significant.

In contrast with the results found on cell extracts, the conditioned media only displayed a 1.2-fold increase on uPA activity upon treatment with 75 μ M histamine, $p < 0.05$ (Figure 3). This activity was 100% inhibited in the conditioned media from cells treated with 1 μ M pyrilamine and 75 μ M histamine, $p < 0.05$.

Type IV collagenase immunoassays on hepatic cells and conditioned media

The immunoassays show that histamine treatment increased the amount of type IV collagenase at all the tested concentrations, both in hepatic cells and their conditioned media. The levels of collagenase in cell extracts were from 3.5- to 4-fold higher than those of the control cells, $p < 0.05$. Again, the highest levels were detected on cells treated with 75 μ M histamine. On the other hand, the amount of total protein on the conditioned media from the treated cells was from 2.5 to 3.8-fold higher than on the conditioned media from the control cells, $p < 0.05$ (Figure 4). Pyrilamine only showed a 50% inhibition of the effect of histamine treatment on cell extracts, but its inhibition for the conditioned media

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was complete (100%), $p < 0.05$. This effect was not observed for cimetidine.

Immunoassays for uPA on hepatic cells and conditioned media

The results from the immunoassays show that the amounts of uPA in the WRL-68 hepatic cells and the conditioned media differ. While the amount of uPA increased on the cell extracts to a level around 4.2-fold higher than the control for all the tested histamine concentrations, it showed a higher than four-fold increase on the conditioned media, $p < 0.05$ (Figure 5). The highest amounts were found on the conditioned medium from cells treated with 75 μM histamine (12-fold). Pyrilamine was absolutely inhibitory (100%) for the effect of histamine, both in cell extracts and conditioned media, $p < 0.05$. This effect could not be detected for cimetidine, due to the intrinsic stimulatory properties of both pyrilamine and cimetidine for the amount of uPA, $p < 0.05$.

It is important to comment that although the amounts of uPA and type IV collagenase were markedly increased in both the extracts and the conditioned media from WRL-68 hepatic cells, this protein was not active, as shown by the zymographic assays.

Discussion

Chronic hepatic disease is characterized by the loss of the homeostatic mechanisms controlling not only the synthesis and deposition of the matrix (fibrogenesis), but its degradation and removal (fibrolysis) [33]. Hepatic fibrosis represents a complex pathophysiological reaction that involves parenchymatous, non-parenchymatous and inflammatory cells, the components of the extracellular matrix and several autocrine, paracrine and juxtacrine signal relay systems [34-36].

One of the current problems of hepatology is the elucidation of the molecular mechanisms involved in the genesis of hepatic fibrosis. Therefore, our group was interested in the study of the role of histamine on the modulation of the synthesis, secretion and activity of type IV collagenase and uPA in the hepatic cell line WRL-68, obtained from normal human hepatic tissue. This cell line has some functional properties that mimic those of hepatocytes *in vivo* [37].

It has been known for more than a decade that histamine can influence the functioning of the normal liver. Histamine forms a complex with cytochrome P450 and is an intracellular modulator of this enzyme in hepatic cells [38]; the presence of histamine deposits in the nucleus of hepatic cells and its involvement in DNA synthesis has been shown [39]; histamine modulates, via H1 receptors, the metabolism of liver cells by increasing the activity of the phosphorylase and the synthesis of phosphatidylinositol [40]; and stimulates glycogenolysis through the activation of the calcium messenger [41].

The involvement of histamine in chronic hepatic disease has been documented decades. First, changes in the content of histamine have been detected during experimental fibrosis, presumably derived from an increase in the number of macrophages on the inflamed area of the liver [42, 43]. Second, hydroxy-proline increases days after histamine increases in the liver,

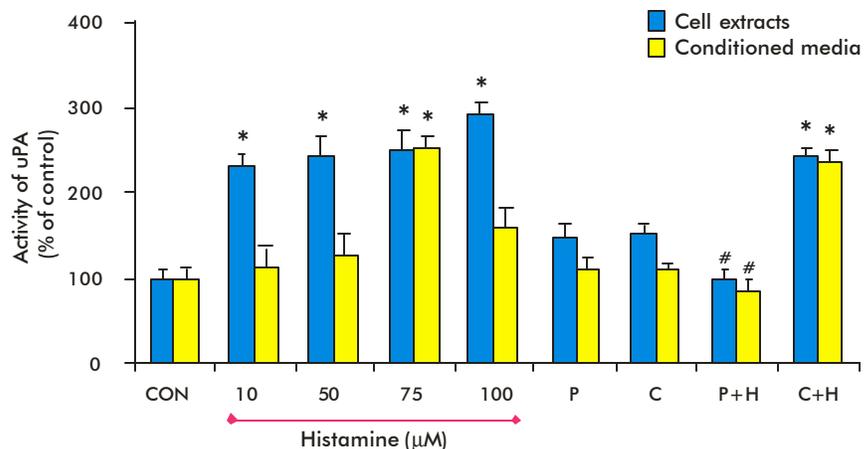


Figure 3. Effect of histamine on uPA activity. The cells were treated with different histamine concentrations for 24 h. Pyrilamine (P) at 1 μM or cimetidine (C) at 1 mM, both H1 and H2 antagonists respectively, were added together with 75 μM histamine (H) or alone. Protein samples containing 20 μg were electrophoresed and analyzed by zymography. The density of the bands was quantified with imaging software, and the results were expressed as a percentage relative to the control. The bars with a different symbol are significantly different from the control group (* $p < 0.05$) or from the group treated with 75 μM histamine (# $p < 0.05$).

which also suggests the possibility that histamine might participate in collagen biosynthesis [18]. Third, high levels of plasmatic histamine have been found in patients with different forms of hepatic disease; and there are significant changes on the histamine receptors, together with high concentrations of endogenous histamine, in patients and in animal models for cirrhosis [44-47].

Histamine has been shown to regulate several events of the inflammatory process; not only as a chemical mediator for its early stages [48-50], but also as a biosynthetic modulator and in the healing processes found during chronic inflammation [51].

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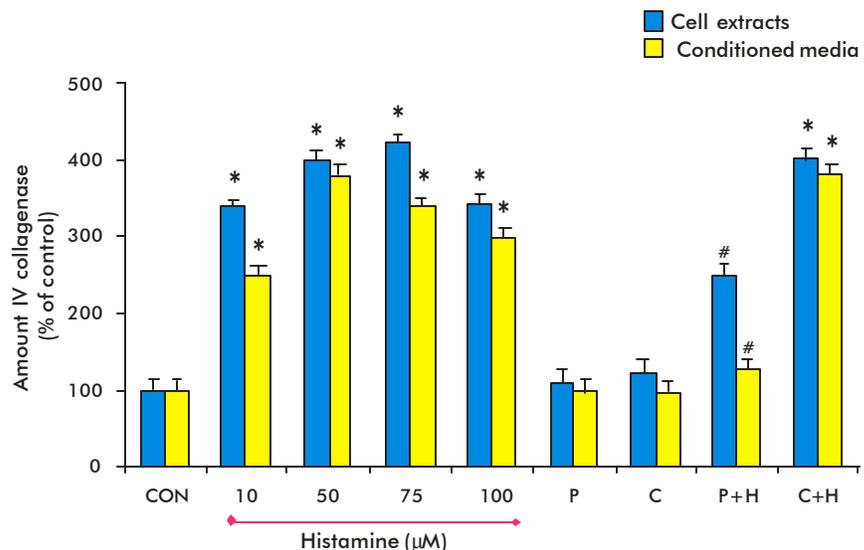


Figure 4. Effect of histamine on the amount of type IV collagenase. Cells were treated for 24 h. with different histamine concentrations. Pyrilamine (P) at 1 μM or Cimetidine (C) at 1 mM, which are H1 and H2 antagonists respectively, were added simultaneously with 75 μM histamine (H) or alone. Samples of cell extracts and conditioned media were subjected to an immunoassay using polyclonal antibodies against type IV collagenase. The results are expressed as a percentage relative to the control group. The bars with a different symbol are significantly different from the control group (* $p < 0.05$) or from the group treated with 75 μM histamine (# $p < 0.05$).

Previous studies have shown that histamine can modulate the proteolytic activity in chronic inflammatory diseases. For example, it has been proven that histamine stimulates the synthesis of metalloprotease-1 in fibroblasts during rheumatoid diseases in humans [52], as well as the synthesis of metalloprotease-3, metalloprotease 13, TNF alpha and matrix prostaglandin E₂ in chondrocytes, and metalloprotease 1, metalloprotease 3 and prostaglandin E₂ in synovial fibroblasts. This suggests that histamine plays a physiopathological role in the tissue remodeling that occurs in osteoarthritis [53]. The published literature shows that PMN leukocytes from ethanol-treated rats have a high capacity for the activation of latent collagenase, and that this effect is partially inhibited by histamine receptor antagonists, implying that histamine might be implicated in the process of activation of latent collagenase [54]. Although histamine can modulate the activity of liver collagenase via PMN leukocytes, there are thus far no reports about the role of histamine on the regulation of the proteolytic activity of hepatic cells.

Previous studies have shown that hepatocytes express both H1 and H2 histamine receptors [55, 56]. However, the plasma membrane from hepatocytes has a greater proportion of H1 receptors, and [H³]-pyrilamine is a representative antagonist for high affinity H1 receptors [57]. Our results show that pyrilamine, but not cimetidine, completely inhibited the synthesis and the activity of both type IV collagenase and uPA. Therefore, histamine may affect hepatic cells through H1, rather than H2 receptors. The data presented here coincides with previous reports where it has been shown that histamine stimulates the proteolytic activity of chondrocytes and fibroblasts via the H1 receptor [49, 53].

In this study, we show that histamine increases the proteolytic activity of a cell line from human hepatocytes (WRL-68). While the activity of type IV collagenase was increased only by the treatment with histamine at 75 µM, uPA activity increased at every tested concentration. Although histamine induces the synthesis, secretion and activity of type IV collagenase and uPA in WRL-68 cells, most of the collagenase secreted to the conditioned medium is inactive. The data show high amounts of type IV collagenase and uPA in either cell extracts or conditioned media, suggesting that histamine may potentially regulate the synthesis, activity and secretion of proteases in the WRL-68 hepatic cell line.

By inducing both synthesis and proteolytic activity, histamine might thus regulate a complete and powerful mechanism for the degradation of the extracellular matrix during hepatic injury. However,

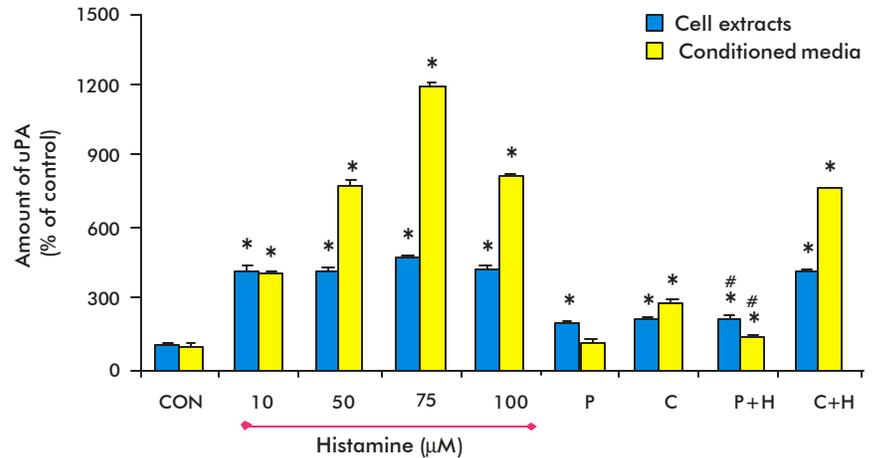


Figure 5. Effect of histamine on the amount of uPA. Cells were treated for 24 h. with different histamine concentrations. Pylamine (P) at 1 µM or Cimetidine (C) at 1 mM, which are H1 and H2 antagonists respectively, were added simultaneously with 75 µM histamine (H) or alone. Samples of cell extracts and conditioned media were subjected to an immunoassay using polyclonal antibodies against uPA. The results are expressed as a percentage relative to the control group. The bars with a different symbol are significantly different from the control group (*p<0.05) or from the group treated with 75 µM histamine (# p<0.05).

the absence of proteolytic activity on the conditioned media suggests that histamine might also induce the synthesis of collagenase and uPA inhibitors, or other molecules, in this cell line. Hellstrand *et al.* have pointed out that a low histamine level in blood correlates with a poor response to interferon treatment in hepatitis C [58]. In this case, the perpetuation of fibrosis may be a consequence of the effects of histamine on the hepatic cells.

Histamine is a versatile mediator that, according to *in vitro* studies, affects the synthesis of inflammatory cytokines and acute phase proteins via the H1 receptor [59-62]. It is acknowledged that after hepatic injury several inflammatory infiltrate or resident cell types may be involved in the synthesis and release of soluble factors that influence the biological function of several cell lineages. This means that a group of soluble factors (cytokines and/or growth factors), specifically aimed at different target cells, is temporarily present in the damaged tissue. Probably none of these factors function alone, but rather belong to a complex network of interactions between the mediators and their targets. Consequently, the possibility of an *in vivo* interaction between histamine and other mediators, which can influence the proteolytic activity of the hepatocytes and, specifically, of the stellate cell, can not be dismissed.

Further studies will be necessary for a better understanding of the role of histamine in fibrogenesis.

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