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Inhibition of heparin synthesis by methotrexate in rats *in vivo*

Sisi Marcondes[a,*], Elaine C. Baú[b], Edson Antunes[a], Carl P. Dietrich[b], Helena B. Nader[b], Gilberto De Nucci[a]

[a]*Department of Pharmacology, Faculty of Medical Sciences, UNICAMP, P.O. Box 6111, 13081-970, Campinas (SP), Brazil*
[b]*Departamento de Bioquímica, UNIFESP, Escola Paulista de Medicina, 04044-020, São Paulo (SP), Brazil*

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Abstract

The content and synthesis of heparin and mast cell-dependent skin oedema (as an indirect evaluation of histamine and serotonin content) were investigated in the rat skin after chronic treatment with compound 48/80, a mast cell degranulating substance. The effect of methotrexate, a folic acid analogue that interrupts the synthesis of DNA and RNA, on heparin synthesis and amine storage also was evaluated in rat skin. The heparin content at 6 and 240 hr after treatment with compound 48/80 was reduced markedly (86 and 64%, respectively). At 6 hr, heparin synthesis increased 3.1-fold compared with control animals; maximal synthesis occurred at 24 hr post-treatment (12.8-fold increase), decaying at 240 hr (2.4-fold increase). The dermatan sulfate content and synthesis were not affected by treatment with compound 48/80. Autoradiographic analysis revealed that methotrexate (2.5 mg/kg for 3 consecutive days) abolished heparin synthesis at 6, 24, and 72 hr after compound 48/80 treatment, without affecting dermatan sulfate synthesis. The oedema induced by intradermal injection of compound 48/80 (1 μg/site) into the rat skin was decreased significantly at 6 hr after chronic treatment with this compound, but was restored completely 72 hr post-treatment. This pattern of oedematogenic response was also observed in the methotrexate-treated rats. In conclusion, our results show that methotrexate suppresses heparin synthesis without affecting the synthesis of either dermatan sulfate or the co-stored amines histamine/serotonin (as evaluated by measuring the mast cell-dependent oedema), suggesting that the enzyme system involved in heparin synthesis is inducible.

Keywords: Mast cells; Proteoglycans; Heparin synthesis; Compound 48/80; Methotrexate; Inflammation

1. Introduction

Mast cells are distributed throughout connective tissues in the body, but are especially abundant adjacent to blood and lymphatic vessels and nerves, and beneath epithelial surfaces, such as those of the respiratory and gastrointestinal systems as well as the skin [1]. These cells synthesise and release a number of mediators that act on vascular and non-vascular smooth muscle, connective tissue, mucous glands, and inflammatory cells [2–4]. These mediators include lipid-derived substances (prostaglandin D_2, leukotriene C_4, and platelet-activating factor), preformed mediators (histamine, serotonin, and neutral proteases), cytokines, and chemokines [5]. Additionally, mast cells are known to synthesise and store proteoglycans including heparin [6,7], and chondroitin sulfate B [8] and E [9–11]. Mast cells are frequently grouped into either mucosal or connective tissue subtypes. These subtypes are distinguishable by morphological, histochemical, and biochemical characteristics, as well as by function and role(s) in inflammation and immunity [12]. Oversulfated proteoglycans are useful phenotypic markers for distinguishing the subclasses of mast cells. Connective tissue mast cells contain heparin, whereas the mucosal mass cells contain chondroitin sulfate. These proteoglycans are co-stored in mast cells with the oedema-forming amines histamine and serotonin, and upon activation by immunological and non-immunological stimuli, they are released into the extracellular space.

Heparin synthesis, which occurs mainly in mast cells, is a complex multistep process that is initiated by the sequential addition of four monosaccharides, namely xylose,

* Corresponding author. Tel.: +55-19-3788-7185; fax: +55-19-3289-2968.
E-mail address: s_marcondes@uol.com.br (S. Marcondes).

Abbreviations: TCA, trichloroacetic acid; PDA, 1,3-diaminopropane acetate buffer; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; GAG, glycosaminoglycan.

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galactose, galactose, and glucuronic acid (linker tetrasaccharide), to the protein core by specific glycosyltransferases. The sugar chains are extended by the addition to this linker of two alternating monosaccharides, an aminosugar and a glucuronic acid. In heparin and heparan sulfate, the aminosugar is *N*-acetyl-glucosamine, whereas in chondroitin sulfate and dermatan sulfate it is *N*-acetyl-galactosamine. The extent of epimerisation of glucuronic acid to iduronic acid and the sulphatation pattern of the disaccharide units distinguish heparin from heparan sulfate, and dermatan sulfate from chondroitin sulfate [13,14]. The heparin synthesis has been studied largely using cultured cells, but no studies have focussed on the *in vivo* mechanisms regulating its synthesis. In addition, although animal skin such as that of rats and humans is usually rich in mast cells [15], most current studies have employed isolated peritoneal mast cells. Therefore, we investigated the content and synthesis of heparin and mast cell-dependent skin oedema (as an indirect evaluation of histamine and serotonin content) in rat skin mast cells after prolonged treatment with compound 48/80, a mast cell degranulating substance. Both heparin synthesis and amine storage in skin mast cells were also evaluated in animals treated with methotrexate, a folic acid analogue that interrupts DNA and RNA synthesis. The present study shows that methotrexate inhibited heparin synthesis but did not interfere with dermatan or histamine synthesis, suggesting that expression of the enzymes involved in heparin synthesis is inducible.

2. Materials and methods

2.1. Materials

Compound 48/80 and chondroitin 4/6-sulfate (from bovine trachea) were purchased from Sigma Chemical Co. ^{125}I-Human serum albumin and sodium pentobarbitone (Sagatal) were obtained from Amersham International and Rhone Merieux, respectively. Heparan sulfate (from bovine pancreas), prepared by quaternary amine complexation, and dermatan sulfate (from bovine intestinal mucosa) were supplied by Opocrin Laboratories. Heparin (from bovine intestinal mucosa) was obtained from Laboratório Opoterápico Brasileiro Ltda.; agarose (Standard Low-M_r) from Bio-Rad Laboratories; 1,3-diaminopropane, 1,2-diaminoethane, and isobutyric acid from the Aldrich Chemical Co.; toluidine blue from the Fisher Scientific Co.; and Cetavlon from Merck. Whatman No. 1 paper was purchased from W & R Balston Ltd. Heparinase from *Flavobacterium heparinum* was prepared as previously described by Nader *et al.* [16], and maxatase (alkaline protease P 126) was purchased from Biocon do Brasil Industrial Ltda. Methotrexate was obtained from Bristol Laboratories. [^{35}S]Sulfate was obtained from the Instituto de Pesquisas Energéticas Nucleares (IPEN).

2.2. Animals

Experiments were carried out on male Wistar rats (180–200 g) obtained from the Central Animal House (CEMIB) of the State University of Campinas (UNICAMP) in accordance with the guidelines for animal care of UNICAMP.

2.3. Depletion of histamine and 5-hydroxytryptamine

The rats were depleted of their stores of histamine and serotonin by repeated injections of compound 48/80, a classical mast cell degranulator, as previously described [17]. Compound 48/80 dissolved in sterile saline (0.9%) was given i.p. morning and evening for eight doses, starting with an evening dose. The dose employed was 0.6 mg/kg for the first three injections and 1.2 mg/kg for the last five injections. Control animals were injected with sterile saline instead of compound 48/80. The efficacy of this treatment was examined by the ability of compound 48/80 itself to induce rat hind-paw oedema, using a hydroplethysmometer (model 7150).

2.4. Methotrexate treatment

Rats were treated with methotrexate (2.5 mg/kg, i.p., daily) for 3 consecutive days, and used 3 days after the last injection of this compound [17]. In animals receiving methotrexate and compound 48/80 concomitantly, the experimental procedures consisted of injecting a single dose of methotrexate on days 1 and 2. On day 3, the animals received both drugs, while on the last 3 days rats were injected with compound 48/80 alone. The efficiency of the methotrexate treatment was confirmed by a significant reduction of the number of peripheral leukocytes (40%; $P < 0.05$) in the treated animals.

2.5. Determination of heparin content and synthesis in the rat dorsal skin

The heparin content in the rat dorsal skin was determined at different time intervals (6–240 hr) after stopping treatment with compound 48/80. Briefly, [^{35}S]sulfate (500 μCi) was injected i.p. 6 hr before the rats were killed. The dorsal skin was removed, and the heparin content was determined by electrophoresis.

2.6. Extraction of glycosaminoglycans

The rat skins were cut into small pieces and homogenized in 10 vol. of acetone for 18 hr at 5°. The mixture was centrifuged (3000 *g* at 4° for 10 min) and dried under vacuum. Acetone powder (1 g) was suspended in 10 mL of 0.05 M Tris–HCl buffer, pH 8.0, in the presence of 0.5 M NaCl. The suspension was incubated with 10 mg of maxatase, for 18 hr at 60° with agitation. After incubation, contaminants present in this suspension were removed with

10% trichloroacetic acid (TCA) for 15 min at 4°. The supernatant was precipitated with 2 vol. of methanol for 18 hr at −20°. The extraction of glycosaminoglycans in the precipitate was achieved by a slight modification of the procedure of Toledo and Dietrich [18]. Briefly, 2 M anhydrous potassium acetate was added to the precipitate, and the mixture was shaken to ensure complete dissolution of the salt. The pH was adjusted to 5.7, and the suspension was maintained in an oven for 2 hr at 60°. About 100 mg of Celite was added to the suspension that was then filtered at 60°. The filtrate was collected in a centrifuge tube and kept at 4° overnight. The precipitate was collected by centrifugation (5000 *g* for 120 min at 4°) and dissolved in 100 μL of water. Ethanol (2 vol.) was added to this solution. After standing overnight in the cold, the precipitate formed was collected by centrifugation (5000 *g* for 30 min at 4°) and dried. It was then dissolved in 100 μL of water and analysed for heparin. To the supernatant was added 2 vol. of ethanol. The precipitate formed was collected by centrifugation (5000 *g*, at 4° for 30 min), dried, resuspended in 1 mL of water, and analysed. The recovery of sulfated glycosaminoglycans by this procedure was comparable to a previously used method [19]. This method has the advantage of separating heparin from the other sulfated glycosaminoglycans. Recovery experiments with heparin and the other sulfated glycosaminoglycans have shown that only heparin precipitates with potassium acetate, and all the other sulfated glycosaminoglycans remain in the supernatant free of the heparin [20]. The limit of detection of heparin by use of this procedure was 0.5 μg/g of dry tissue.

2.7. Electrophoresis

Electrophoresis was performed in agarose (0.55%) as described by Jaques *et al.* [21] and modified by Dietrich and Dietrich [22], in either 0.05 M 1,3-diaminopropane–acetate buffer (PDA), pH 9.0 [23] or a discontinuous system using 0.04 M barium acetate, pH 5.8, and PDA, pH 9.0 [24]. The compounds were precipitated using 0.1% Cetavlon for 2 hr at room temperature and, after drying, the gel was stained with 0.1% toluidine blue in 1% acetic acid and 50% ethanol. The glycosaminoglycans were quantified by densitometry at 525 nm of the agarose slides. The error of the method was in the order of about 4.5%. The extinction coefficients of the glycosaminoglycans were calculated using standards of chondroitin 4-sulfate, dermatan sulfate, heparan sulfate, and heparin. ^{35}S-Labeled glycosaminoglycans were visualized after exposing stained agarose gels to a Kodak SB-5 film for 10–20 days. The film was then quantified by densitometry.

2.8. Degradation by heparinase

Aliquots of 100 μg of rat skin heparin and intestinal heparin were incubated with 0.05 units of heparinase in 0.05 M ethylenediamine–acetate buffer (pH 7.0) containing 0.02 M calcium acetate, at 30° in a final volume of 30 μL for 18 hr. The incubation mixtures were then applied to Whatman No. 1 paper and subjected to chromatography in isobutyric acid:1.25 M NH_3 (5:3.6, v/v) for 18 hr. The products were visualized with the aid of a short wave UV lamp and staining with 0.1% toluidine blue in absolute ethanol [25].

2.9. Measurement of local oedema formation in rat skin

Local oedema was measured in male Wistar rats (180–220 g) as the local accumulation of i.v. injected ^{125}I-human serum albumin into skin sites [26]. Rats were anaesthetized by i.p. injection of sodium pentobarbitone (Sagatal, 30–40 mg/kg). ^{125}I-Human serum albumin (10 μCi/kg) and Evans blue dye (1.5 mL/kg, 2.5%, w/v) were injected i.v. Compound 48/80 was made up in Tyrode bicarbonate solution and injected in volumes of 100 μL into the shaved dorsal skin according to a balanced site pattern with two replicates. After a 30-min accumulation period, a 5-mL cardiac blood sample was taken, and the animals were killed by cervical dislocation. The dorsal skin was removed, and the injection sites were punched out (15 mm diameter) and counted for radioactivity in a γ-counter. Oedema formation at each site was expressed as plasma volume, calculated from the counts in 1 mL of plasma.

2.10. Statistical analysis

Results of skin oedema were analysed by ANOVA followed by Bonferroni's modified *t*-test. A $P < 0.05$ was considered to be significant.

3. Results

3.1. Content and synthesis of heparin and dermatan sulfate in dorsal skin of rats treated chronically with compound 48/80

The content of proteoglycans in the dorsal skin was determined at 6 and 240 hr after compound 48/80 treatment. Figure 1A shows that heparin content was decreased by 86% at 6 hr post-treatment compared with the control animals. At 240 hr post-treatment, a significant reduction of heparin content was observed, but this reduction was less evident than at 6 hr (Fig. 1A), indicating a partial recovery of the heparin content. The enzymatic degradation of either rat skin heparin or standard heparin (from bovine intestinal mucosa) by heparinase resulted in the appearance of both trisulphatede disaccharide and tetradisaccharide, confirming the identity of heparin rather than other glycosaminoglycans (Fig. 1B). In contrast to heparin, the dermatan sulfate content in treated animals did not differ from that of control rats at any time evaluated

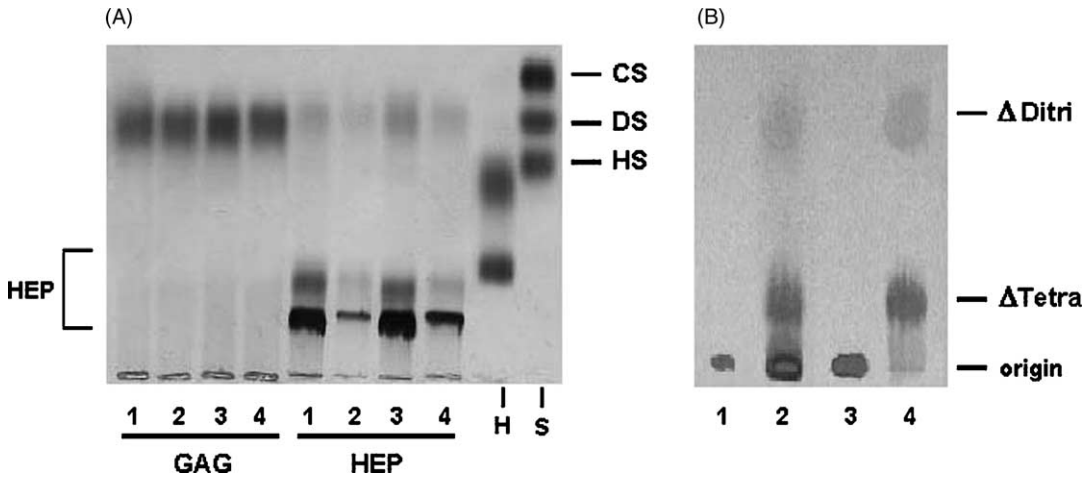

Fig. 1. Agarose gel electrophoresis of glycosaminoglycans extracted from rat skin of control and 48/80-treated animals. Aliquots (5 μL) of heparin (HEP) and glycosaminoglycan fractions (GAG) obtained from rat skin after potassium acetate precipitation were applied to an agarose gel slab (10.0 × 7.5 × 0.2 cm) in 0.04 M barium acetate buffer, pH 5.8, and subjected to electrophoresis in a discontinuous 0.04 M barium acetate buffer, pH 5.8/0.05 M 1,3-diaminopropane–acetate buffer, pH 9.0, buffer system. Panel A: (lane 1) control, 6 hr after saline injection; (lane 2) treated, 6 hr after compound 48/80 treatment; (lane 3) control, 240 hr after saline injection; (lane 4) treated, 240 hr after compound 48/80 treatment; (H) 5 μg of standard intestinal heparin; and (S) standard mixture of glycosaminoglycans containing chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS). Panel B: enzymatic degradation of either rat skin heparin or standard heparin (from bovine intestinal mucosa) by heparinase. (Lanes 1 and 2) standard bovine heparin in the absence and presence of heparinase, respectively; (lanes 3 and 4), rat skin heparin content in the presence and absence of heparinase, respectively. (ΔDitri) trisulphatede disaccharide and (ΔTetra) tetradisaccharide. This figure is a representative result from three individual experiments.

(Fig. 1A). With regard to chondroitin and heparan sulfate, no detectable presence of these proteoglycans was observed (Fig. 1A).

Heparin and dermatan sulfate synthesis in both control and compound 48/80-treated animals was also studied. In control rats, heparin synthesis was very low, while at 6 hr post-treatment it increased 3.1-fold as compared with the control animals (Fig. 2B). The maximal heparin synthesis occurred at 24 hr post-treatment (12.8-fold increase, Fig. 2B), decaying at 240 hr (2.4-fold increase). As opposed to heparin, a clear dermatan sulfate synthesis was observed in control rats, and this was not modified by compound 48/80 at any time studied post-treatment (Fig. 2B).

3.2. Effect of methotrexate on heparin content and synthesis

Treatment of rats with methotrexate in the absence of compound 48/80 did not affect the content of heparin in the rat skin compared with control animals receiving saline (Fig. 3). Heparin synthesis was not observed in either of these groups (Fig. 2B).

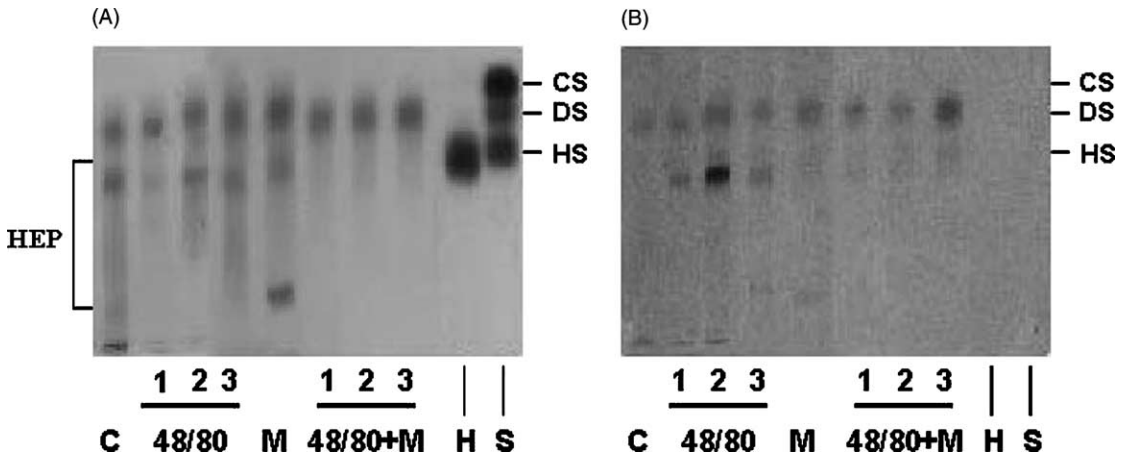

Fig. 2. Agarose gel electrophoresis of skin glycosaminoglycans from rats treated with methotrexate and compound 48/80. Aliquots (5 μL) of the GAG extracted from the skin of different rats by TCA were applied to an agarose gel slab (10.0 × 7.5 × 0.2 cm) in 0.05 M 1,3-diaminopropane–acetate buffer, pH 9.0, and subjected to electrophoresis. After staining with toluidine blue (panel A), the gel was exposed to an x-ray film (panel B). (C; control) 6 hr after saline injection; (lanes 1, 2, and 3) 6, 24, and 72 hr, respectively, after compound 48/80 treatment in the presence or absence of methotrexate (M). (H) 5 μg of standard intestinal heparin and (S) a standard mixture of GAG containing chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS). This figure is a representative result from three individual experiments.

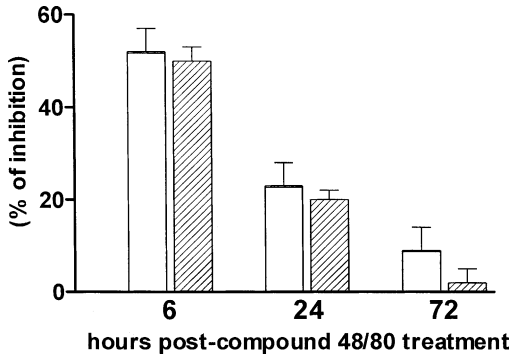

Fig. 3. Effect of methotrexate on rat skin oedema induced by intradermal injection of compound 48/80 (1 μg/site) at 6, 24, and 72 hr after chronic degranulation of mast cells *in vivo*. Compound 48/80 and methotrexate treatments were carried out simultaneously as stated in Section 2. Open and hatched columns represent oedema inhibition (%) after compound 48/80 treatment in the absence and presence of methotrexate, respectively. Results are means ± SEM (N = 3–5).

In rats treated with both methotrexate and compound 48/80, heparin content was reduced at 6 hr post-treatment; however, in contrast to animals treated with compound 48/80 alone, no recovery was observed at 24 and 72 hr (Fig. 2A). In addition, analysis by autoradiography revealed that methotrexate virtually abolished heparin synthesis in rats treated with compound 48/80, as determined at 6, 24, and 72 hr (Fig. 2B). On the other hand, the simultaneous treatment with methotrexate and compound 48/80 affected neither the content nor the synthesis of dermatan sulfate compared with that of control animals (Fig. 2, A and B).

3.3. Rat skin oedema

The injection of compound 48/80 (1 μg/site) into skin sites of control rats induced significant oedema (95 ± 4 μL) compared with Tyrode-injected sites (20 ± 2.0 μL; $P < 0.001$). In animals treated chronically with compound 48/80, skin oedema in response to intradermal injection of compound 48/80 was reduced by 52 ± 5% ($P < 0.001$) and 24 ± 4% ($P < 0.01$) at 6 and 24 hr post-treatment, respectively. At 72 hr, compound 48/80-induced skin oedema did not differ significantly between control and 48/80-treated rats (Fig. 3). Treatment of the animals with methotrexate had no significant effect on oedema formation in control or compound 48/80-treated animals (Fig. 3).

4. Discussion

In this study, a prolonged treatment of rats with compound 48/80 in the presence or absence of methotrexate was performed, and both *de novo* synthesis and content of heparin in mast cells of rat dorsal skin were examined at different time intervals post-treatment. Our results clearly showed a marked decrease in the heparin content in the mast cells of the skin at 6 hr after compound 48/80 treatment. At 240 hr post-treatment, heparin content increased, but did not reach the same amount as the control group. Furthermore, the incorporation of [^{35}S]sulfate indicated that the synthesis of heparin by mast cells of the skin peaked at 72 hr after post-treatment, decaying thereafter (240 hr). In agreement with our study, the heparin content of peritoneal mast cells has been shown to normalise 72 hr after treatment of rats with a single dose of compound 48/80 [27]. Interestingly, an *in vitro* study showed a complete recovery of both heparin content and synthesis as early as 6 hr after incubation of isolated peritoneal mast cells with compound 48/80 [28]. These results suggest that after complete depletion, heparin synthesis *in vivo* is much slower than in isolated cells, which is consistent with the idea that endogenous factors down-regulate the synthesis of this proteoglycan. It has been reported that some interleukins such as interleukin-1α, interleukin-1β, and interleukin-11, tumour necrosis factor α, oncostatin M, ciliary neurotrophic factor, and leukaemia inhibitory factor inhibit proteoglycan synthesis [29–32]. It is also interesting to note that in control rats not only were the synthesis and content of dermatan sulfate clearly detected but they also were not modified by compound 48/80 treatment. Conversely, heparin synthesis in control animals was almost undetectable, becoming remarkable in conditions of complete mast cell degranulation. Since dermatan sulfate is a structural proteoglycan [33] whereas heparin [34] is stored in the mast cell granules, one may speculate that heparin synthesis takes place only in conditions of cell activation.

Methotrexate, a folic acid analogue, is an immunosuppressive drug that acts by interrupting DNA and RNA syntheses [35,36]. Our findings showed that recovery of heparin content after treatment with compound 48/80 was not observed in animals treated simultaneously with methotrexate as a consequence of heparin synthesis abrogation. Furthermore, methotrexate did not affect dermatan sulfate synthesis, indicating a very specific effect on the heparin metabolic pathway.

Heparin is stored together with the amines histamine and serotonin in mast cells. When given intradermally, compound 48/80 induces local mast cell degranulation leading to the release of the vasoactive mediators histamine and serotonin, which in turn increase vascular permeability and cause local oedema formation. Thus, in rats chronically treated with compound 48/80, we attempted to evaluate the recovery of skin oedema in response to intradermal injection of compound 48/80 itself. Our results showed that skin oedema was largely reduced at 6 hr post-treatment and partially restored at 24 hr. A full recovery was observed at 72 hr, indicating by this time a re-establishment of histamine and serotonin contents necessary to evoke oedema formation. In a previous study, the passive cutaneous anaphylaxis response in rats chronically treated with compound 48/80 was abolished immediately after treatment and partially recovered 2 weeks later [37]. In isolated mast

cells depleted of their stores of histamine/serotonin, histidine decarboxylase activity (the enzyme responsible for histamine synthesis) was restored after 20 min of incubation with compound 48/80 [28]. Although a discrepancy regarding the time necessary to recover the amine contents in mast cells after complete depletion could be noticed between the *in vivo* and *in vitro* studies, a close relationship between heparin content and serotonin/histamine recovery was observed in both studies. It is known that chronic treatment with compound 48/80 depletes mast cells of their stored amines (histamine and serotonin) without affecting the number of dermal mast cells [38]. This may exclude the possibility that reduction of skin oedema and heparin synthesis in rats treated chronically with compound 48/80 was due to a reduction of mast cell numbers at the cutaneous microcirculation. Additionally, our findings that methotrexate treatment suppressed heparin synthesis without affecting dorsal skin oedema induced by intradermal injection of compound 48/80 are indicative that the number of mast cells was not changed and suggest that the histamine and/or serotonin metabolic pathways may not be modified in situations where heparin synthesis is suppressed.

In conclusion, our results have provided a good experimental model to study the mechanisms that regulate *de novo* heparin synthesis *in vivo*. Furthermore, our findings that methotrexate suppresses heparin synthesis without affecting the synthesis of either dermatan sulfate or the co-stored amines histamine/serotonin suggest that the enzyme system involved in heparin synthesis is inducible rather than constitutive.

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