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Respective role of lipoxygenase and nitric oxide-synthase pathways in plasma histamine-induced macromolecular leakage in conscious hamsters

*,[1]G. Gimeno, [2]P.H. Carpentier, [1]S. Desquand-Billiald, [1]M. Finet & [1]R. Hanf

[1]Service de Pharmacologie, Laboratoire Innothéra, 10 av PV Couturier, BP 35, 94111 Arcueil cedex, France; [2]Laboratoire de Médecine Vasculaire, Pavillon Elysée Chatin, CHRU Grenoble, 38043 Grenoble cedex, France

1 Intravital microscopy technique was used to determine the distribution of a fluorescent plasma marker (fluorescein-isothiocyanate-dextran, 150 kD; FD-150) into venular and interstitial compartments of dorsal skin fold preparations in conscious hamsters.

2 One mg kg^{-1} histamine (i.v.) caused a biphasic decrease in venular fluorescence due to FD-150 extravasation in all organs (general extravasation). Immediately after injection, the venular fluorescence decreased and plateaued in 60 min. Ninety minutes after histamine injection, venular fluorescence further decreased until 180 min. Prior treatment with indomethacin (0.1 mg kg^{-1}, i.v.) did not modify the time-course of general extravasation but prevented histamine-induced venule dilatation.

3 Prior treatment with the 5-lipoxygenase activating protein (FLAP) inhibitor, 3-[1-(p-chlorobenzyl)-5-(isopropyl)-3-t-butylthioindol-2-yl]-2,2-dimethyl-propanoic acid sodium (MK-886)(10 μg kg^{-1}, i.v.), the leukotriene receptor antagonist, benzenemethanol a-pentyl-3-(2-quinolinylmethoxy) (REV-5901)(1 mg kg^{-1}, i.v.), or the glutathione-S-transferase inhibitor, ethacrynic acid (1 mg kg^{-1}, i.v.), delayed by 60 min the onset of general extravasation caused by 1 mg kg^{-1} histamine.

4 Prior treatment with lipoxygenase pathway inhibitors and N^G-nitro-L-arginine-methylester (L-NAME)(100 mg kg^{-1}, i.v.) abolished the general extravasation and venule dilatation induced by 1 mg kg^{-1} histamine.

5 Injection of 1 μg kg^{-1} (i.v.), of leukotriene-C_4 (LTC_4) or -D_4 (LTD_4) induced immediate and sustained general extravasation and reduction in venule diameter, these effects being blocked by REV-5901.

6 Histamine (1 mg kg^{-1}, i.v.) induced biphasic decline in mean arterial blood pressure (MAP). An initial phase (from 0 to 60 min) was followed by a late phase beginning 90 min after histamine injection. L-NAME (100 mg kg^{-1}, i.v.) and aminoguanidine (1 mg kg^{-1}, i.v.) prevented the late phase of histamine-induced hypotension.

7 Thus, plasma histamine can trigger both an immediate cysteinyl-leukotriene (Cys-LT)-dependent and a late nitric oxide (NO)-mediated inflammatory cascade. Although the cyclo-oxygenase (COX) pathway might account for histamine-induced venule dilatation, it would not influence histamine-induced extravasation.

Keywords: Cyclo-oxygenase; 5-lipoxygenase; nitric oxide; leukotrienes; permeability

Abbreviations: MK-886, 3-[1-(p-chlorobenzyl)-5-(isopropyl)-3-t-butylthioindol-2-yl]-2,2-dimethyl-propanoic acid, sodium; REV-5901, Benzenemethanol a-pentyl-3-(2-quinolinylmethoxy); CCD, Charge-coupled device; COX, Cyclo-oxygenase; Cys-LT, Cysteinyl-leukotriene; DMSO, Dimethyl sulphoxide; FLAP, Five-lipoxygenase activating protein; FD-150, Fluorescein-isothiocyanate-dextran, 150 kD; iNOS, Inducible NO-synthase; LTB_4, Leukotriene-B_4; LTC_4, Leukotriene-C_4; LTD_4, Leukotriene-D_4; LTE_4, Leukotriene-E_4; MAP, Mean arterial blood pressure; L-NMMA, N^G-monomethyl-L-arginine; L-NAME, N^G-nitro-L-arginine-methyl-ester; NO, Nitric oxide; PAF, Platelet activating factor; PMN, Polymorphonuclear leukocytes

Introduction

Histamine induces macromolecular extravasation and polymorphonuclear leukocytes (PMN) adhesion. Suffused histamine permeabilizes the exteriorized organs due to activation of the endothelial histamine H_1 receptor and the subsequent formation of gaps between endothelial cells (Fox *et al*., 1980; Wu & Baldwin, 1992; Yuan *et al*., 1993). This direct permeabilizing effect is transient (peak at 3–5 min, total recovery 15–20 min after the onset of histamine suffusion) and is inhibited by the L-arginine analogues, L-NAME or N^G-monomethyl-L-arginine (L-NMMA) (Mayhan, 1994; Paul *et al*., 1994). All published studies of histamine suffusion are consistent with these being a phospholipase C-NO-cyclic GMP-dependent macromolecular leakage.

We have recently shown (Gimeno *et al*., 1998a), that bolus injection of histamine at doses of above 0.01 mg kg^{-1} causes biphasic macromolecular extravasation in the microcirculation of conscious hamsters. There is an initial phase which develops fully within 15–60 min and is followed by a late, NO-mediated, PMN adhesion-dependent extravasation beginning 90 min after histamine injection. The late phase is inhibited by L-NAME, L-NMMA and aminoguanidine, the selective inhibitor of the inducible form of NO-synthase. In contrast, the initial phase is insensitive to NO-synthase inhibition, ruling out direct

*Author for correspondence; E-mail: inno3@club-internet.fr

permeabilization of the endothelium by histamine. The inflammatory process involved in the early phase of plasma histamine-induced macromolecular extravasation is poorly understood. It may involve white cell activation and liberation of inflammatory mediators acting in synergy to cause rapid NO-independent leakage. Thus, cysteinyl-leukotrienes (LTC_4, LTD_4 and LTE_4), leukocyte-derived inflammatory mediators, rapidly induce macromolecular extravasation if suffused onto hamster cheek pouch (Dahlen *et al*., 1981; Mayhan, 1993).

In this study we investigated the involvement of cyclo-oxygenase, lipoxygenase and NO-synthase pathways in the two phases of plasma histamine-induced macromolecular extravasation using hamster dorsal skin fold preparations and intravital microscopy. The dorsal skin fold preparation (Endrich *et al*., 1980) makes it possible to observe the skin microcirculation in conscious animals for prolonged periods of time (up to 4 weeks). It also avoids the effects of acute inflammatory reactions caused by surgical procedures and the effects of anaesthetics on the microcirculation. The use of this technique with adapted computer-assisted image analysis makes possible the long-term study of the effects of plasma histamine on macromolecular extravasation.

Methods

Surgical procedure

A titanium window chamber facilitating observation of the dorsal skin microcirculation was surgically implanted into the skin back of anaesthetized (pentobarbitone, 60 mg kg^{-1}) male Syrian golden hamsters (6–8-weeks-old; weight 60–80 g) as described by Endrich *et al*., (1980). Two titanium frames were sewn on so as to sandwich an extended double layer of dorsal skin from which the hair had been removed. Once the first frame of the chamber was fixed, the upper layer of skin in contact with it was completely removed over a defined circular area (15 mm diameter). The exposed layer was moistened with 0.9% saline solution and covered with a cover slip fixed to the second frame. A subcutaneous venous catheter was inserted into the jugular vein, positioned on the dorsal side of the neck and sewn to the frames. Dorsal skin microcirculation was then observed through a defined area within the frames and various drugs were systemically applied *via* the catheter. The animals were allowed to recover from anaesthesia and surgery for 48 h. There was no sign of discomfort, such as changes in sleeping or feeding habits during this recovery period. Hamster dorsal skin fold preparations are quiescent with respect to the endothelial barrier function because no inflammatory changes are observed in the striated muscle tissue contained in the chamber, as assessed by both light and electron microscopy (Endrich *et al*., 1980).

Experimental protocol

Experiments were performed 2 days after surgery, in a dark, quiet and temperature-controlled (21°C) laboratory. Conscious animals were placed in a specially designed cylinder which enabled the window of the chamber to be positioned perpendicular to the light beam of a Leitz Ergolux microscope (Leica, France). A fluorescent macromolecular tracer (FD-150; 63 mg kg^{-1}) was administered *via* the jugular catheter 15 min before initiation of inflammation by bolus injection of histamine (0.01 or 1 mg kg^{-1}) or leukotriene (LTB_4, LTC_4 or LTD_4; 1 μg kg^{-1}) through the same catheter. In some animals, a COX inhibitor (indomethacin; 0.1 mg kg^{-1}), a FLAP inhibitor (MK-886; 0.01 mg kg^{-1}), a glutathion-S-tranferase–LTC_4 synthase inhibitor (ethacrynic acid; 1 mg kg^{-1}), a 5-lipoxygenase inhibitor–Cys-LT receptor antagonist (REV-5901; 1 mg kg^{-1}), NO-synthase inhibitors (L-NAME, 100 mg kg^{-1} or aminoguanidine 1 mg kg^{-1}) or appropriate vehicle was injected 15 min before inflammatory mediator to block COX, 5-lipoxygenase or NO-synthase pathways, respectively. The doses of inhibitors derived from previously published results demonstrating selective effects in experiments performed in several animal species (Leung, 1986; Hogaboam *et al*., 1992; Lehr *et al*., 1993; Gimeno *et al*., 1998a).

The time course of leukotriene-induced or histamine-induced macromolecular extravasation was determined by analysing FD-150 fluorescence distribution in the dorsal skin preparation every 15 min during 180 min experiments. For that purpose, the preparation was sequentially epi-illuminated (for 1 min every 15 min) at the optimal excitation wavelength for FD-150 (450–490 nm) using a 100-W mercury bulb and appropriate filters (I_3 blue block, Leica) positioned between the light source and the condenser. A black and white charge-coupled device (CCD) video camera (HPR 610, Instrumentation Capteur SA, Meylan, France) was connected to the microscope and images were sent to a VHS video-recorder (Mitsubishi BV-2000E, Bioblock, France) for off-line computer-assisted image analysis.

Computer-assisted image analysis

After injection of FD-150, epi-illumination of the preparation made it possible to observe the venules and arterioles in the skin microcirculation. The analogue video images collected during the experiments were digitized into x, y arrays of 512 by 512 pixels (picture element). Each pixel was associated with an 8-bit grey scale level between 0 (black) and 255 (white) as previously described (Bekker *et al*., 1989). A grey scale histogram was then constructed by scanning the entire digitized image. Two or three gaussian curves (Microcal Origin software) were fitted to the histogram, demonstrating that pixels were normally distributed in two main homogeneous populations along the grey scale axis. On average, at $t=0$ (i.e. just before injection of histamine or leukotriene), these peaks were centred on positions 81 ± 7 and 177 ± 6 of the 256 attributable grey scale levels. The high grey level peak accounted for the brighter population of pixels corresponding to venules whereas the low grey level peak accounted for the darker population of interstitial pixels. An additional third minor peak could be visualized when an arteriole was included into the image. Such analysis of the fluorescence distribution over the whole image was possible because fluorescence variations observed in response to inflammatory mediators were uniform in both venular and interstitial compartments. This allowed all the venular and interstitial pixels to be taken into account for a rapid (less than 10 s $image^{-1}$) and precise (maximal number of pixels) statistical determination of the mean fluorescence intensity in the venular and interstitial compartments of the image. We have never seen leaky site appearence in response to plasma histamine or leukotriene whether post-capillary venules were included in the image or not. This could introduce regional variation in the fluorescence of the interstitial compartment and in turn could distort the darker peak.

Measurements of drug-induced macromolecular extravasation

The injection of saline solution at $t=0$ min had no significant effect on fluorescence distribution in these 3 h experiments. In

contrast, interstitial fluorescence was higher and venular fluorescence lower than initial levels, 180 min after bolus injection of 1 mg kg^{-1} histamine. Amplitude of the darker peak shift has been assumed to be proportional to the amount of FD-150 lost by the vasculature in all organs (general extravasation) since in preliminary calibration experiments without inflammatory mediator, venular mean grey level was proportional to the dose of FD-150 injected (from 20–140 mg kg^{-1}). Furthermore, the histamine-induced decrease in venular fluorescence could not be related to enhanced concentration of red blood cells since haematocrit measured at $t=180$ min were not significantly different in histamine- and saline-treated animals. For determination of the time course of histamine- or leukotriene-induced general extravasation, venular peak shift amplitude was determined every 15 min after injection of the inflammatory mediator, throughout 180 min experiments. It was normalized with the difference between venular and interstitial mean grey scale levels at $t=0$ (X_T), refered as 'general extravasation index' and expressed as a percentage of X_T.

The same calculations have been concomitantly performed using the shift amplitude of the brighter peak which accounted for FD-150 accumulation in the interstitial compartment of the skin (local extravasation index). Such analysis of the local extravasation index gave results qualitatively similar to those obtained regarding general extravasation index. Because inflammatory mediators were intravenously-injected, we feel more appropriate to report only the data obtained with general extravasation index.

Venule diameter measurements

Plasma histamine produced general vasodilatation and hypotension. In order to determine whether histamine-induced general extravasation could be associated with a histamine-induced decrease in venular shear rate, diameter of the skin venules was measured on the digitized images. For each image, a grey scale threshold was defined between interstitial and venular grey scale levels. Pixels with grey scale values above this threshold were automatically converted to black (grey scale level 0), those with grey scale values below this threshold were converted to white (grey scale level 255). Therefore, the vascular compartment was shown in black and the interstitial compartment in white. A segment perpendicular to the longitudinal axis of the vessel was defined and Visicap software was used to determine the number of black pixels along this segment and to convert it into micrometres. The collecting venules had an initial diameter of 93 ± 10 μm. Drug-induced changes in venule diameter were measured every 15 min and were expressed as a percentage of the initial diameter.

Mean arterial blood pressure measurements

We were unable to measure arterial blood pressure in conscious hamsters. Blood clots rapidly collapsed the catheters inserted into the left carotid artery during window chamber implantation. Male Syrian golden hamster (12–14-weeks-old; weight 120–150 g) were therefore anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.). For this set of experiments, hamsters older and bigger than those for intravital microscopy were used to facilitate catheterization of the left carotid. A cannula was inserted into the left carotid artery (PE-10, Clay Adams, NJ, U.S.A.) for measurement of mean arterial blood pressure and a cannula was inserted into the right jugular vein (S54-HL, Bioblock, France) for the systemic administration of drugs. Anaesthesia was maintained by bolus administration of pentobarbitone. Drug-induced changes in mean arterial blood pressure were measured every 15 min during 180 min experiments.

Drugs

Fluorescein-Isothiocyanate Dextran (150,000 mw, FD-150), histamine, pyrilamine, cimetidine, aminoguanidine, N^G-nitro-L-arginine methyl ester (L-NAME), indomethacin and ethacrynic acid were dissolved in 0.9% NaCl. Benzenemethanol a-pentyl-3-(2-quinolinylmethoxy) (REV-5901) and 3-[1-(p-chlorobenzyl)-5-(isopropyl)-3-t-butylthioindol-2-yl]-2,2-dimethyl-propanoic acid, sodium (MK-886) were dissolved in dimethyl sulphoxide (DMSO) and NaCl 0.9% (1 : 1000 vol:vol). Leukotriene B_4, C_4, and D_4 were dissolved in ethanol and NaCl 0.9% (1 : 1000 vol:vol). MK-886 was purchased from Calbiochem (Meudon, France). REV-5901, leukotrienes B_4, C_4, D_4 were purchased from Spi-Bio (Massy, France). All other drugs were purchased from Sigma Chemical (Saint-Quentin Fallavier, France). Drugs were intravenously-injected as a bolus *via* the jugular vein.

Statistical analysis

Results were expressed as means $\pm$ s.e.mean. Analysis of variance was performed (ANOVA, Statistica, Statsoft) and statistical significance assessed using Scheffe's *post hoc* test. P values <0.05 were considered to be significant.

Results

Effects of lipoxygenase and cyclo-oxygenase inhibitors on histamine-induced extravasation

As previously described in our previous report, after histamine injection, the general extravasation index increased immediately and reached a plateau after 60–90 min (Figure 1a). A late phase of general extravasation began 90 min after histamine injection and lasted until the end of the experiment. Inhibition of the COX pathway by indomethacin (0.1 mg kg^{-1}) before histamine injection did not affect the time-course of histamine-induced extravasation (Figure 1a). The same results were obtained using mefenamic acid as COX inhibitor (data not shown). The FLAP inhibitor, MK-886 (10 μg kg^{-1}) (Rouzer *et al*., 1990; Hogaboam *et al*., 1992), injected 15 min before histamine, delayed the onset of histamine-induced extravasation (Figure 1b). In the presence of this leukotriene synthesis inhibitor, general extravasation began 60 min after histamine injection and reached a plateau at $t=120$ min. At the end of the experiments, the histamine-induced general extravasation was approximately 30% lower with MK-886 administration. In control experiments (without histamine) indomethacin ($n=5$) and MK-886 ($n=5$) caused no change in venular fluorescence (4.17 ± 1.32% for indomethacin and 7.34 ± 1.88% for MK-886, not significantly different from their respective vehicles). Histamine also caused venular dilatation which could be prevented by 0.1 mg kg^{-1} indomethacin (Figure 1c). MK-886 (0.01 mg kg^{-1}) had no effect on histamine-induced venular dilatation. In control experiments (without histamine) the COX and FLAP inhibitors did not significantly affect venule diameter. In our initial paper, we demonstrated that 0.01 mg kg^{-1} histamine caused an immediate increase in general extravasation index which reached a plateau at $t=60$ min and then remained stable until the end

of the experiments. Using this dose of histamine, the FLAP inhibitor did not delay the onset of extravasation but totally prevented it (Figure 2). At the end of the experiment the general extravasation index measured in the presence of MK-886 was not significantly different from that measured in control experiments performed without histamine ($5.52 \pm 0.59\%$ vs $4.53 \pm 1.63\%$).

Effects of cys-LT synthesis inhibitor and receptor antagonist on histamine-induced extravasation

We investigated the involvement of cys-LT in the general extravasation caused by 1 mg kg^{-1} histamine, animals were pretreated with a cys-LT receptor antagonist, REV-5901 (1 mg kg^{-1}, i.v.), (Van Inwegen *et al.*, 1987; Hogaboam *et al.*, 1992) or with an inhibitor of LTC_4-synthase, ethacrynic acid (1 mg kg^{-1}, i.v.) (Leung, 1986). As for MK-886, treatment with REV-5901 and ethacrynic acid did delay by 60 min the onset of general extravasation induced by 1 mg kg^{-1} histamine (Figure 3a and b). In these experiments, REV-5901 and ethacrynic acid had no effect on histamine-induced venular dilatation (Figure 3c). In control experiments (without histamine injection), REV-5901 and ethacrynic acid neither affected venular fluorescence nor caused change in venule diameter (data not shown). The remaining extravasation observed with 1 mg kg^{-1} histamine after treatment with ethacrynic acid or REV 5901 was presumably mediated by NO because treatment with REV-5901 + L-NAME or ethacrynic acid + L-NAME abolished histamine-induced general extravasation (Figure 3a and b). The general extravasation indexes for these treatments determined 180 min after histamine injection were not significantly different from those measured with vehicle alone ($5.77 \pm 1.12\%$ for REV-5901 + L-NAME; $9.14 \pm 1.58\%$ for ethacrynic acid + L-NAME and $4.53 \pm 1.63\%$ without histamine). In control experiments (without histamine), the non-specific inhibitor of NO-synthases, L-NAME, caused general extravasation (Gimeno *et al.*, 1998b).

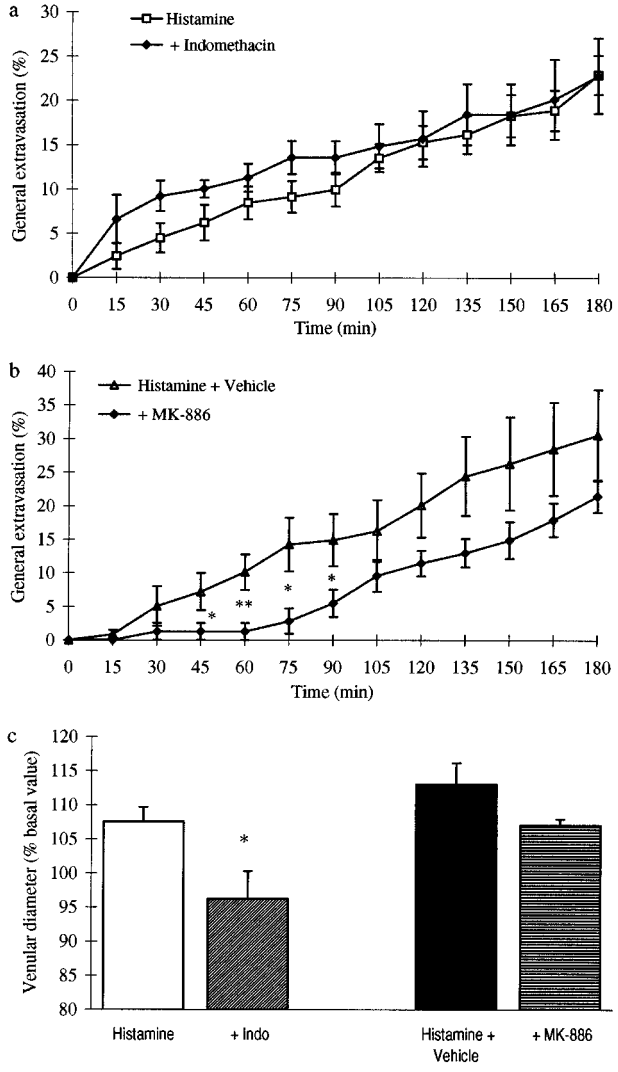

Figure 1 Effects of prior treatment, 15 min before bolus administration of histamine, with the cyclo-oxygenase inhibitor, indomethacin (0.1 mg kg^{-1}, i.v., $n=5$), the 5-lipoxygenase inhibitor, MK-886 (10 μg kg^{-1}, i.v., $n=8$) or vehicle (DMSO 1 : 100 in 0.9% NaCl, $n=6$) on the time-course of general extravasation induced by histamine (1 mg kg^{-1}, i.v., $n=9$) (a and b) and venule diameter changes after 180 min (c). *Denotes a significant difference from value with 1 mg kg^{-1} histamine and #denotes a significant difference from value with 1 mg kg^{-1} histamine previously treated with vehicle (*#$P<0.05$; **##$P<0.01$; ***$P<0.001$) according to Scheffe's *post-hoc* test.

Temporal involvement of lipoxygenase and NO-synthase pathways

Histamine-induced extravasation was evaluated by measuring absolute changes in venular fluorescence over the first 90 min and then over the remaining 90 min of the experiment (Figure 4a). A similar sequential analysis was performed for histamine-induced venular dilatation (Figure 4c). All the drug treatments that inhibited cys-LT synthesis or antagonized leukotriene effects prevented histamine-induced general extravasation over the first 90 min without affecting histamine-induced venular dilatation (Figure 4a). Histamine-induced general extravasation, as well as histamine-induced venular dilatation, between $t=90$ and $t=180$ min was not significantly different from that measured with histamine alone (Figure 4a and c). Additional prior teatment with the non-specific inhibitor of NO-synthases, L-NAME, did not further reduced histamine-induced general extravasation between $t=0$ and $t=90$ min. However, it suppressed the general extravasation which developed between $t=90$ and $t=180$ min in the sole presence of MK-886 and REV-5901 (Figure 4b). In the concomitant presence of

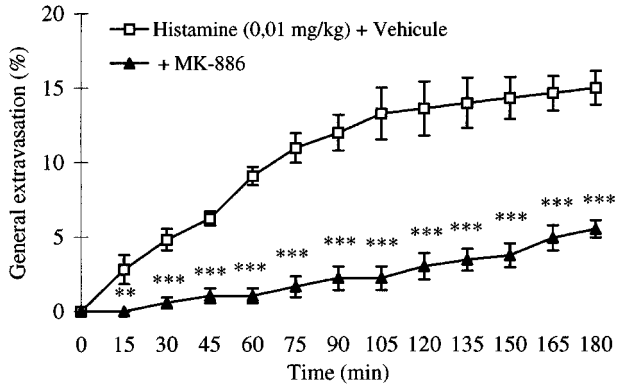

Figure 2 Effects of prior treatment, 15 min before bolus administration of histamine (0.01 mg kg^{-1}, i.v., $n=6$), with the 5-lipoxygenase inhibitor, MK-886 (10 μg kg^{-1}, i.v., $n=8$) or vehicle (DMSO 1 : 100 in 0.9% NaCl, $n=6$) on the time-course of general extravasation. *Denotes a significant difference from value obtained for control preparations (**$P<0.01$; ***$P<0.001$) according to Scheffe's *post-hoc* test.

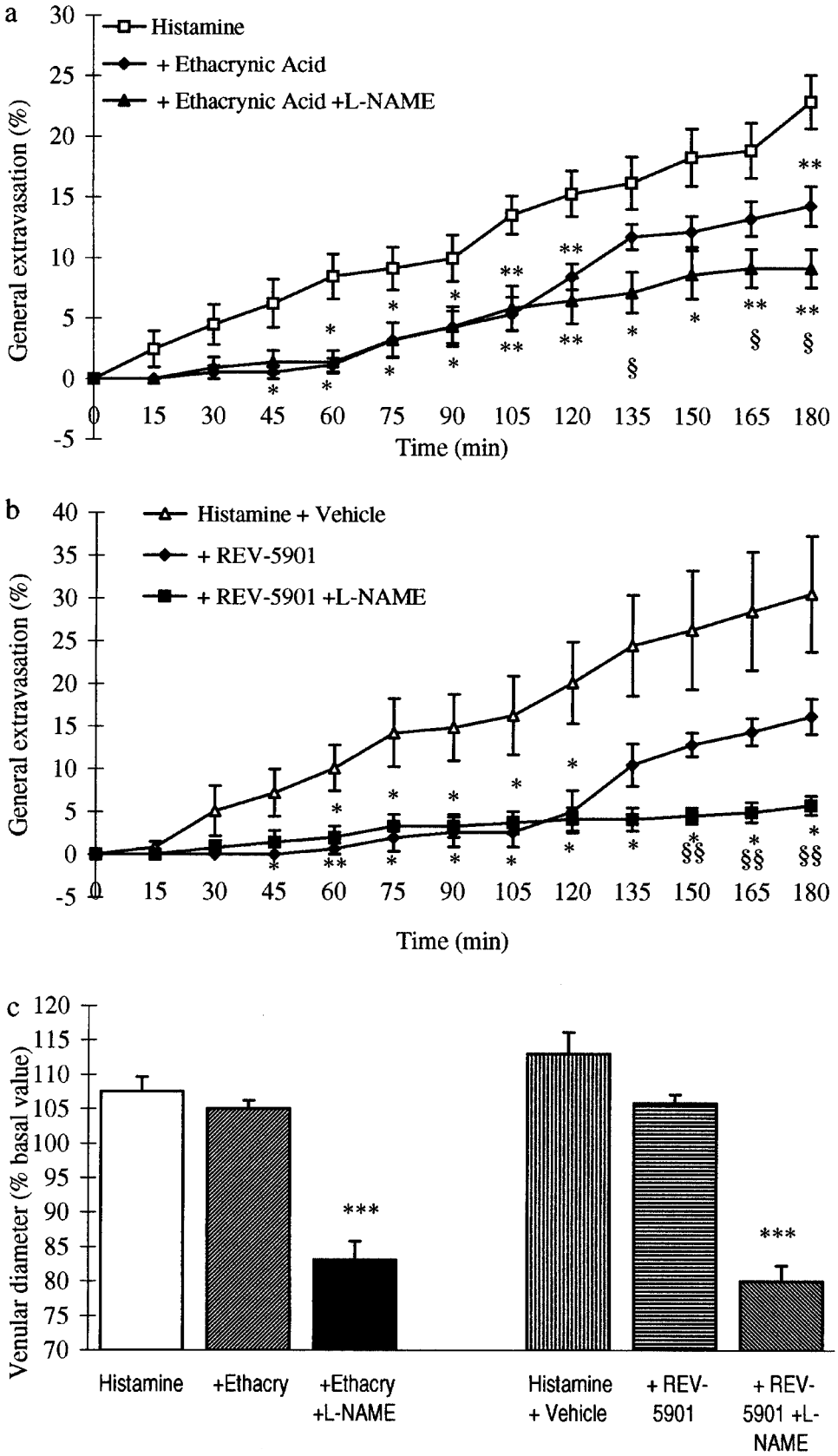

Figure 3 Effects of prior treatment, 15 min before bolus administration of histamine, with the gluthatione S-transferase inhibitor, ethacrynic acid (1 mg kg^{-1}, i.v., $n=7$), or the cysteinyl-leukotriene receptor antagonist, REV-5901 (1 mg kg^{-1}, i.v., $n=6$), alone or in combination with L-NAME (100 mg kg^{-1}, i.v., $n=5$ and 6) on the time-course of general extravasation (a and b) and venule diameter changes (after 180 min) (c) caused by histamine (1 mg kg^{-1}, i.v., $n=9$). *Denotes a significant difference from value obtained for control preparations and § denotes a significant difference from values obtained with preparations pretreated without L-NAME (*§$P<0.05$; **§§$P<0.01$; ***$P<0.001$) according to Scheffe's *post-hoc* test.

leukotriene and NO-synthase inhibitors, histamine did not cause venular dilatation during both periods (Figure 4d). Additional pretreatment with 1 mg kg^{-1} aminoguanidine had a similar effect than L-NAME but failed to prevent histamine-induced venular dilatation between $t=0$ and $t=90$ min (Figure 4b and d).

Leukotriene-induced extravasation

The effects of systemic application of LTB_4, LTC_4 and LTD_4 on macromolecular extravasation were assessed in hamster dorsal skin fold preparations. As expected, the cys-LT, LTC_4 and LTD_4 (1 μg kg^{-1}, i.v.) caused immediate and sustained increase in general extravasation index in 180 min experiments (Figure 5a). They also caused a persistent reduction in venule diameter (Figure 5c). All the effects of LTC_4 were prevented by prior treatment with the cys-LT receptor antagonist, REV-5901 (1 mg kg^{-1}, i.v.) (Figure 5b and c), but not by treatment with the FLAP inhibitor, MK-886 (10 μg kg^{-1}, i.v.) (data not shown). Bolus injection of the chemotactic leukotriene, LTB_4 had no significant effect on venular fluorescence or venule diameter (Figure 5b and c).

Effects of histamine on mean arterial blood pressure

Because macromolecular extravasation is highly dependent on pre-capillary pressure, and because bolus injection of histamine caused generalized vasodilatation and hypotension, we investigated the involvement of COX, lipoxygenase and NO-synthase products in the histamine-induced hypotension. Bolus injection of 1 mg kg^{-1} histamine resulted in biphasic hypotension in anaesthetized hamsters (Figure 6a). A rapid decrease in MAP occurred within the first hour of the experiments. This was further enhanced by a late hypotensive effect which began 90 min after histamine injection. Prior treatments with indomethacin or with MK-886 did not affect the amplitude and the time-course of histamine-induced hypotension (data not shown). Because of its action on endothelial NO-synthase, L-NAME induced sustained hypertension for at least 90 min (data not shown). Consistent with these control experiments, L-NAME increased MAP during the pretreatment period (Figure 6a). Nevertheless, such L-NAME prior treatment did not affect the amplitude of the histamine-induced hypotension between $t=0$ and $t=90$ min but totally prevented histamine-induced decline in MAP

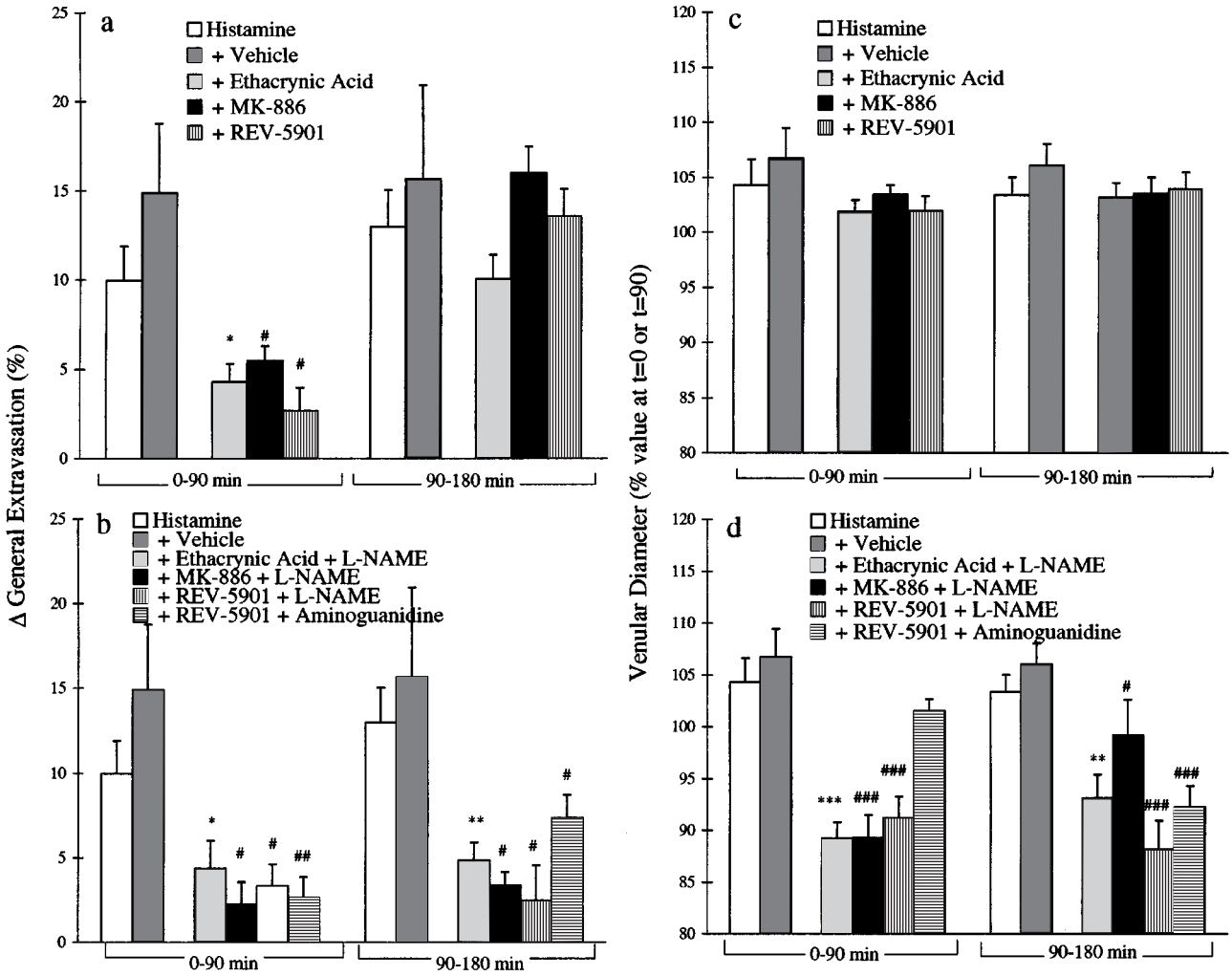

Figure 4 Sequential analysis of the effects of a prior treatment with ethacrynic acid (1 mg kg^{-1}, i.v., $n=7$), REV-5901 (1 mg kg^{-1}, i.v., $n=6$) or MK-886 (10 μg kg^{-1}, i.v., $n=8$), alone or in combination with L-NAME (100 mg kg^{-1}, i.v.; $n=4-6$) or aminoguanidine (1 mg kg^{-1}, i.v.; $n=8$) on general extravasation (a and b) and venule diameter changes (c and d) induced by histamine (1 mg kg^{-1}, i.v.) during the first 90 min and the last 90 min of the experiment. *Denotes a significant difference from the value with 1 mg kg^{-1} histamine and #denotes a significant difference from the value with 1 mg kg^{-1} histamine previously treated with vehicle (*#$P<0.05$; **$P<0.01$; ***###$P<0.001$) according to Scheffe's *post-hoc* test.

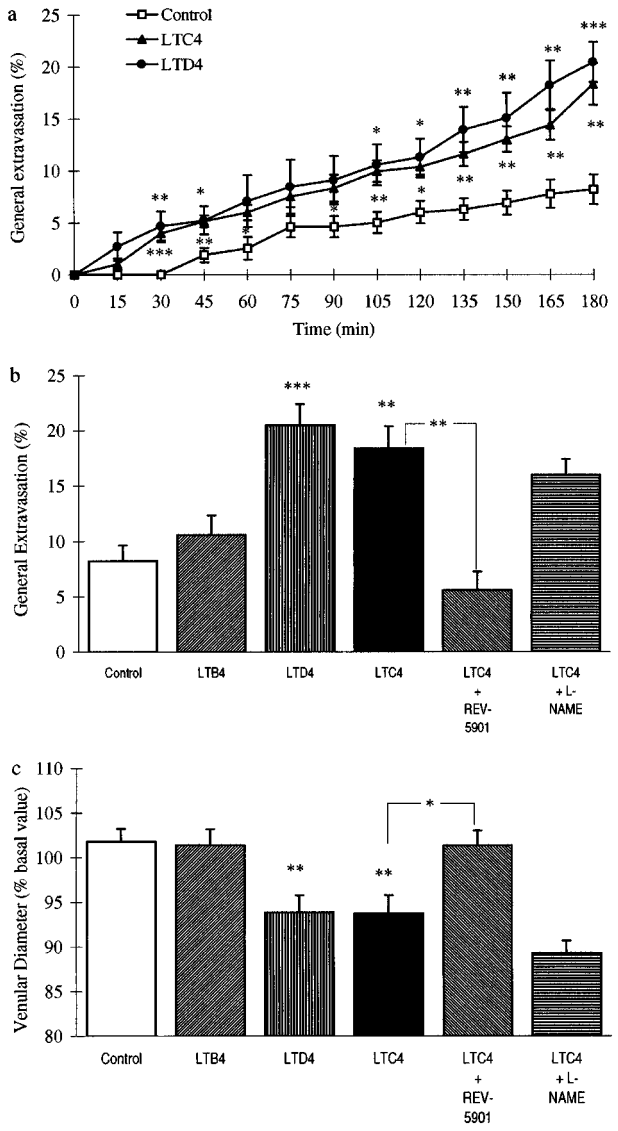

Figure 5 Effects of bolus administration of LTB_4 (1 μg kg^{-1}, $n=6$), LTC_4 (1 μg kg^{-1}, $n=8$) or LTD_4 (1 μg kg^{-1}, $n=6$) on general extravasation (a) and on venular diameter (b). Effects of LTC_4 were also evaluated after a treatment with the cysteinyl-leukotriene receptor antagonist (REV-5901, 1 mg kg^{-1}, i.v., $n=4$) or the non-specific NO-synthase inhibitor, L-NAME (100 mg kg^{-1}, i.v.; $n=5$). *Denotes a significant difference from the value obtained for control preparations ($n=7$) (Scheffe's *post-hoc* test: *$P<0.05$; **$P<0.01$; ***$P<0.001$).

between $t=90$ and $t=180$ min. Aminoguanidine (1 mg kg^{-1}, i.v.) did not modify initial MAP suggesting that conversely to 100 mg kg^{-1} L-NAME, this dose of aminoguanidine did not inhibit constitutive production of NO (Figure 6a). Interestingly, 1 mg kg^{-1} aminoguanidine prevented late phase of histamine-induced hypotension without affecting the time-course and the amplitude of histamine-induced hypotension during the first 90 min of the experiment (Figure 6b). In order to determine the histamine receptor subtype involved in histamine-induced hypotensive effects, animals were pretreated with an H_1 receptor antagonist (pyrilamine, 1 mg kg^{-1}, i.v.) or with an H_2 receptor antagonist (cimetidine, 1 mg kg^{-1}, i.v.). Pyrilamine, did not alter the time-course of histamine-induced hypotension (Figure 6b). Conversely, cimetidine prevented the early phase of histamine-induced hypotension without significant effect on the delayed phase (Figure 6a and b).

Discussion

Consistent with our previous report (Gimeno *et al*., 1998a), these results provide evidence for biphasic macromolecular extravasation in response to systemic administration of histamine and for the involvement of leukotrienes in this process. Prior treatment with the FLAP inhibitor, MK-886, which inhibits the production of leukotrienes by the 5-lipoxygenase pathway significantly delayed the onset of the inflammatory response to bolus injection of 1 mg kg^{-1} histamine. The glutathione-S-transferase–LTC_4 synthase inhibitor, ethacrynic acid, and the cys-LT receptor antagonist, REV-5901, had similar inhibitory patterns to MK-886 consistent with cys-LT having a more important role than the chemotactic lipoxygenase product, LTB_4. This cys-LT-mediated effect of histamine was occurred early and accounted for the first phase (from 0–90 min) of histamine-induced macromolecular extravasation. Consistent with this cys-LT-dependent process, the administration of LTC_4 and LTD_4 induced immediate macromolecular extravasation in the conscious hamster microcirculation, whereas LTB_4 had no effect. Mayhan (1993) have shown that suffusion of LTC_4 or LTD_4 on hamster cheek pouch causes the immediate formation of leakage sites in the endothelium of post-capillary venules by stimulating an NO-dependent process. However, we found that the inhibition of NO synthesis by L-NAME neither prevented the permeabilizing effects of cys-LTs nor affected the cys-LT-dependent extravasation triggered by histamine injection. Therefore, the *in vivo* permeabilizing mechanism of cys-LTs may depend on the route of administration (i.e. intra- vs extra-vascular). Plasma LT effects in conscious hamsters may be indirect *via* the activation of inflammatory cells and subsequent liberation of various inflammatory mediators.

We have assumed that plasma histamine stimulates the endogenous synthesis of cys-LT, resulting in the initial phase of extravasation. The cellular origin of histamine-induced cys-LT is unknown. However, histamine is known to induce platelet activating factor (PAF) liberation by the endothelium (Lorant *et al*., 1991; Watanabe *et al*., 1991), and PAF is a potent chemotactic factor which may cause the activation of circulating PMN. Furthermore, histamine can stimulate PMN adhesion–activation *via* the rapid expression of P-selectin at the endothelial surface (Lorant *et al*., 1991). In our previous report (Gimeno *et al*., 1998a), we showed that the initial phase of plasma histamine-induced extravasation is independent of PMN adhesion. This rules out the involvement of PMN adhesion in the rapid cys-LT-dependent extravasation triggered by histamine injection. Thus histamine may indirectly stimulate cys-LT synthesis by circulating white cells through endothelial liberation of PAF.

Subsequent to the immediate cys-LT-dependent macromolecular extravasation, histamine doses higher than 0.1 mg kg^{-1} (i.v.) triggered an inflammatory cascade which promotes a late NO-dependent extravasation (Gimeno *et al*., 1998a). A question arose to know whether the initial cys-LT-dependent phase of histamine-induced extravasation was required for the late NO-dependent extravasation to be achieved. Several results argue in favour of two independent inflammatory cascades. (i) In reponse to 1 mg kg^{-1} histamine, the delayed extravasation which remained after inhibition of the lipoxygenase pathway could be totally prevented by NO-synthase inhibitors. (ii) In response to 0.01 mg kg^{-1} histamine, the cys-LT-dependent immediate phase of extravasation fully developed within 60 min but the subsequent late phase did not. Nevertheless, both initial and late phases of histamine-induced extravasation can be

prevented by prior treatment with pyrilamine but not with cimetidine, suggesting that the two temporally distinct phases might be initiated by activation of two distinct population of H_1 receptors.

This initial cys-LT-dependent phase of histamine-induced macromolecular extravasation is independent of the haemodynamic effects of the autacoid. During the first 90 min of the experiments, neither the histamine-induced rise in venule diameter nor histamine-induced hypotension were affected by prior treatments with MK-886, REV-5901 or ethacrynic acid. Histamine-induced hypotension was totally prevented by prior treatment with the H_2 receptor antagonist, cimetidine (1 mg kg^{-1}) but this pretreatment did not affect the time-course of histamine-induced macromolecular extravasation (Gimeno *et al*., 1998a). In contrast, the H_1 receptor antagonist, pyrilamine (1 mg kg^{-1}), inhibited both initial and late phases of histamine-induced macromolecular extravasation but did not affect histamine-induced hypotension. Thus, during the first 90 min after histamine injection, haemodynamic and inflammatory effects are presumably mediated by different histamine receptor subtypes, H_2 and H_1 receptors respectively.

H_2-mediated hypotension during the first 90 min of the experiments was insensitive to the constitutive NO-synthase blockade according to a direct interaction of histamine with the vascular smooth muscle cells. The role of H_1-mediated activation of the endothelium and of subsequent NO-mediated relaxation of the vascular smooth muscle was negligible in the initial phase of histamine-induced hypotension but this activation would account for the NO-mediated rise in venule diameter (this study; Gimeno *et al*., 1998a).

Histamine also triggered an inflammatory cascade which, in turn, stimulated late NO-dependent extravasation. In our previous report, we suggested that this late macromolecular leakage was due to PMN adhesion-dependent expression of the inducible form of NO-synthase, because it was prevented by fucoidan, non-specific inhibitors of NO-synthases (L-NAME; L-NMMA) and low dose (1 mg kg^{-1}) of aminoguanidine, a specific inhibitor of the inducible form of NO-

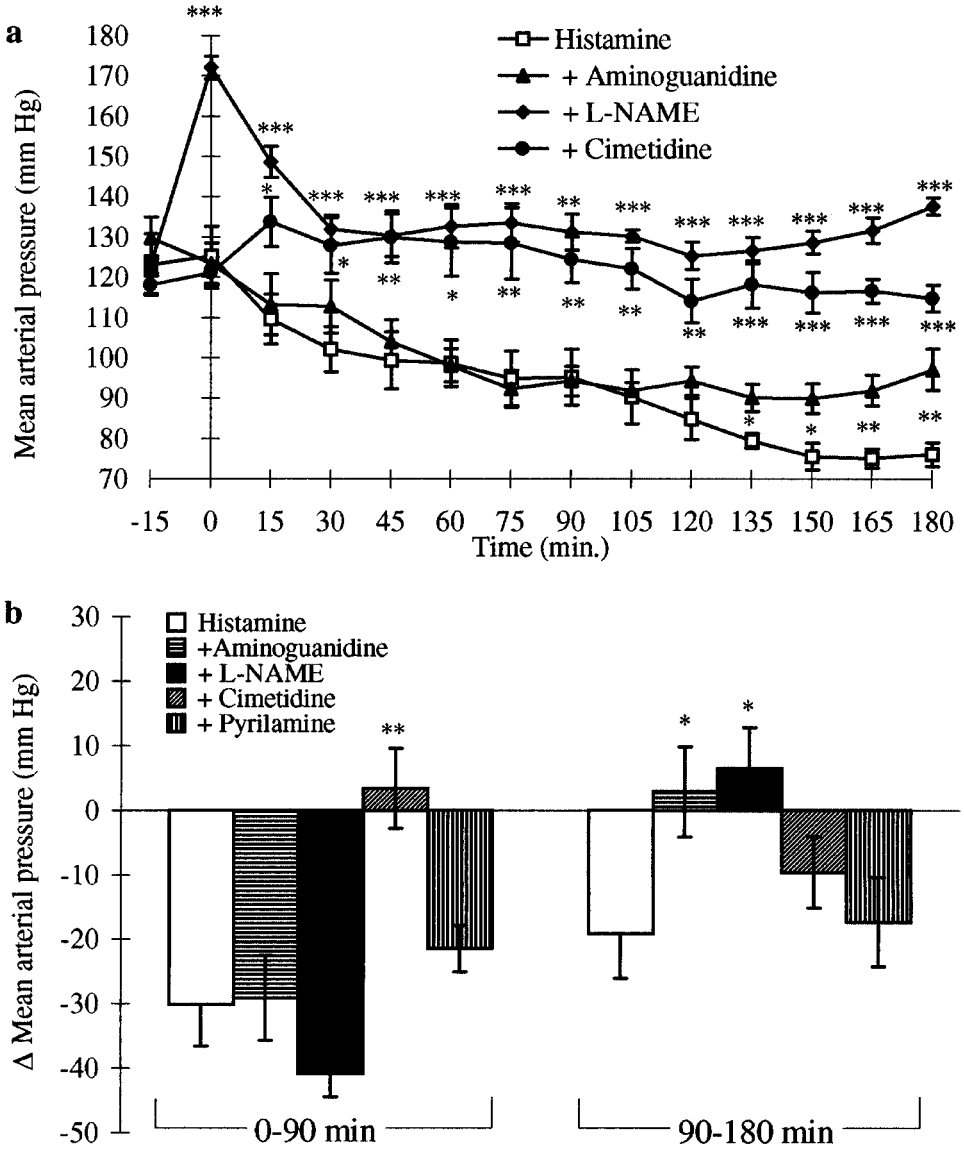

Figure 6 Effects of a prior treatment with specific (aminoguanidine, 1 mg kg^{-1}, $n=6$), or the non-specific (L-NAME, 100 mg kg^{-1}, i.v.; $n=7$), inhibitors of NO-synthase on the mean arterial blood pressure changes induced by histamine (1 mg kg^{-1}, i.v.; $n=8$). The involvement of the H_2 histamine receptor was assessed by a prior treatment with the H_2 histamine receptor antagonist, cimetidine (1 mg kg^{-1}, i.v.; $n=6$). *Denotes a significant difference from the effects of histamine according to Scheffe's *post-hoc* statistical test. (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

synthase (iNOS). In this study, plasma histamine (1 mg kg^{-1}) caused biphasic hypotension, the late hypotensive effect of histamine being prevented by prior treatment with 1 mg kg^{-1} aminoguanidine. This dose of aminoguanidine did not reproduce the hypertensive effects of L-NAME (100 mg kg^{-1}) which result from inhibition of the constitutive endothelial NO-synthase. These experiments provide evidence for late histamine-induced expression of iNOS, responsible for macromolecular extravasation, venule dilatation and hypotension during the 90–180 min period. The late histamine-induced NO-dependent extravasation is prevented by pyrilamine (Gimeno *et al*., 1998a) but the late NO-dependent phase of histamine-induced hypotension is not blocked by this H_1 antagonist (data not shown) nor by the H_2 receptor antagonist cimetidine.

The two phases of plasma histamine-induced extravasation may result from the activation of H_1 receptors, but the autacoid initiates two independent, temporally distinct, inflammatory cascades. The remaining late extravasation observed with inhibition of rapid cys-LT-dependent leakage was totally prevented by treatment with L-NAME or aminoguanidine, suggesting the induction of late NO-dependent extravasation did not require the first phase of extravasation. We previously demonstrated that the two phases of histamine-induced extravasation have different patterns of reactivity to the autacoid. Thus, the two independent inflammatory cascades may be triggered by the activation of two separate populations of H_1 receptors.

Thus, the macromolecular leakage induced by plasma histamine does not involve the direct NO-mediated permeabilizing effect of histamine on the endothelium (Wu & Baldwin, 1992; Yuan *et al*., 1993; Mayhan, 1994; Paul *et al*., 1994). In conscious hamsters, plasma histamine triggers two indirect inflammatory cascades: a rapid cys-LT-dependent cascade and a delayed NO-mediated cascade resulting from late expression of iNOS. These mechanisms may be of critical importance in anaphylactic shock.

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