Involvement of gaseous low molecular monoxides in the cutaneous reverse passive Arthus reaction   - Cytoprotective action of carbon monoxide -

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A running title: CO and NO in the Arthus Reaction

Key words: Nitric oxide; Carbon monoxide; Arthus reaction; Heme oxygenase-1

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SUMMARY

The deposition of immune complexes (IC) induces an acute inflammatory response with tissue injury, for which the involvement of nitric oxide (NO) and carbon monoxide (CO) has been suggested. NO is induced by NO synthase (NOS) and CO is generated by heme oxygenase (HO). Among HO isoenzymes, HO-1 is an induced type. To assess the role of NO and CO in the pathogenic process, the cutaneous reverse passive Arthus reaction was examined using NOS inhibitor, HO-1 stimulator and HO-1 inhibitor. To evaluate the reaction we considered edema, tumor necrosis factor-α, interleukin-6, and neutrophil number. The values of these four parameters were significantly reduced in mice treated with HO-1 stimulator as compared to the positive control mice. Quite the reverse was observed in mice treated with HO-1 inhibitor. These results suggest that HO-1/CO signalling pathway is a therapeutic target for human IC-mediated disease.

INTRODUCTION

The formation and local deposition of immune complexes (IC) are thought to be important factors which induce acute inflammatory responses with significant tissue injury. IC injury has been implicated in a variety of human diseases including vasculitis syndrome, systemic lupus erythematosus, rheumatoid arthritis and glomerulonephritis [1]. The classical and standard animal model for the inflammatory response in these IC-mediated diseases is the Arthus reaction, which results in edema, hemorrhage and neutrophil recruitment in the skin by the intradermal injection of horse serum repeatedly into a rabbit [2]. Because of its ease and reproducibility, the experimental model most commonly used is the reverse passive Arthus reaction, in which an antibody (Ab) is injected at the site where the investigator wants the inflammatory response to develop and the antigen is applied intravenously immediately before or after the antibody injection. As the accumulation of neutrophils, nuclear dusts, fibrin deposits, hemorrhage and edema are recognized in this model, this reverse passive Arthus reaction is thought to be a very good model for vasculitis in the field of dermatology [3, 4].

On the other hand, the involvement of reactive oxygen species (ROSs) has been reported in the pathogenesis of tissue injury in the inflammation [5]. Among ROSs, nitric oxide (NO) is a gaseous radical whose role has been important in the etiology of many diseases [6], ever since NO was found to be an endothelium-derived relaxing factor in 1991 [7]. Furthermore, NO reacts with superoxide, which is also one of the ROSs, and immediately produces peroxynitrite which can cause severe cytotoxicity [8]. NO is produced from L-arginine by NO synthase (NOS), and three isoforms have been identified. Meanwhile, carbon monoxide (CO) is a gaseous molecule, like NO, and is induced by heme oxygenase (HO) in the process of heme metabolism [9]. Recently, CO has been highlighted because of its involvement especially in inflammatory diseases [10]. Both NO and CO are low molecular monoxides and have actions of vasodilatation. In addition, they can activate soluble guanylate cyclase and an increase in the level of cyclic GMP. However, NO is a radical and reported to show severe cytotoxicity, whereas CO is stable and thought to be cytoprotective [11].

With regard to NO, the possibility was reported that endothelial damage was reduced by the inhalation of NO gas during lung transplantation [12], but it was also reported that the clinical application of an NOS inhibitor could control the drop in blood pressure of a patient with septic shock [13]. As regarding CO, Giannini L et al. reported the protection from cardiac injury by induction of HO-1 in a focal ischemia-reperfusion model [14]. Furthermore, the exposure to

CO gas was reported to increase the survival rate in mice which suffered from a septic shock induced by lipopolysaccharide [15]. In short, NO has been implicated in the pathogenesis of diseases and the application of NOS inhibitor has been reported to demonstrate the promise to be able to relieve some conditions of diseases. Furthermore, some papers reported similar promise for HO-1 stimulator. Such evidences lead us to consider that NO seemed to be cytodestrctive and CO seemed to be cytoprotective in most cases [16].

There are several stimulators and inhibitors available with regard to NOS and HO-1, which is an induced type of HO. Therefore, we conducted the present study in order to examine the roles of NO and CO in the reverse passive Arthus reaction using NOS inhibitor, HO-1 stimulator and HO-1 inhibitor.

MATHERIALS AND METHODS

Chemicals

N^G-nitro-L-arginine methyl ester (L-NAME) was adopted as a NOS inhibitor, hemin as a chemical HO-1 stimulator and Zinc protoporphyrin-IX (Znpp-IX) as a competitive HO-1 inhibitor (Sigma-Aldrich, St Louis MO). Ten or 30 mg L-NAME was dissolved in 100 ml phosphate buffered saline (PBS) [17]. 65.2 mg hemin or 62.2 mg Znpp-IX were dissolved in 4 ml of 0.1 mol/l NaOH, followed by titration with 0.1 mol/l HCl and the total volumes were raised to 10 ml by adding sterile distilled water [18].

Mice

The mice used were 12 to 16-week-old female C57BL/6, purchased from the Jackson Laboratory (Bar Harbor, ME), and were healthy, fertile, and did not display evidence of infection or disease. They were housed in a pathogen-free barrier facility and screened regularly for pathogens. All studies and procedures were approved by the Committee on Animal Experimentation of Nagasaki University Graduate School of Biomedical Sciences.

Reverse passive Arthus reaction

For cutaneous reverse passive Arthus reactions, the mice were anesthetized by inhalation of diethyl ether, shaved on their dorsal skins, and wiped with 70% alcohol. Rabbit IgG anti-chicken egg albumin Abs (60mg/30ml; Cappel, Aurora, OH) were injected intradermally with a 29-gauge needle, followed immediately by an intravenous injection of chicken egg albumin (20 mg/Kg; Sigma-Aldrich), which was meant to create a positive IC challenge [19]. The intradermal injection of purified polyclonal rabbit IgG (60 mg/30 ml, Sigma-Aldrich) followed by intravenous introduction of chicken egg albumin served as a negative control. The solution of chicken egg albumin contained 0.4% Evans blue dye (Sigma-Aldrich). A zero control group was established by sacrificing some of the positive control group immediately after positive IC challenge. Besides these negative, positive and zero control groups, four additional groups were created in the present study: 1) 1 mg/kg L-NAME treated group, mice injected intravenously by 1 mg/kg L-NAME just after positive IC challenge; 2) 3 mg/kg L-NAME treated group, mice injected intravenously by 3 mg/kg L-NAME just after positive IC challenge [17]; 3) hemin treated group, mice injected intravenously by 80 mmol/kg hemin just after positive IC challenge;

and 4) Znpp-IX treated group, mice injected intraperitoneally by 100 mmol/Kg Znpp-IX, 48 hours, 24 hours and 1 hour before positive IC challenge [20]. The Znpp-IX treated group of mice was sacrificed at 4 hours after IC challenge and other groups at either 4 hours or 8 hours. The zero control group consisted of 5 mice while each other group consisted of 7 to 10 mice.

Quantification of vasculitis

The degrees of vasculitis were assessed by the levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, edema and neutrophil recruitment in the lesional skins.[4] The levels of TNF-α and IL-6 were measured using enzyme-linked immunosorbent assay (ELISA) kits. Edema was evaluated by measuring the vascular leak. Evans blue dye binds to serum proteins and thereby can be used to quantify alterations in vascular permeability [21]. 100 mg/ml Evans blue solution was made and then standard series were produced by two-fold dilution from 50 mg/ml to 0.4 mg/ml. The absorbances of samples and standard series were measured at 620 nm using a spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan), and the concentration of Evans blue dye in each sample was calculated against the calibration curve of the standard Evans blue solution. The measured values were divided by respective sample weights and corrected values were used for statistical analysis.

Histological examination

Tissues were harvested 0, 4 or 8 hours after IC challenge using a disposable sterile 6-mm biopsy (Maruho, Osaka, Japan). The excised tissues were cut into halves and one half was fixed in 4% paraformaldehyde, and then paraffin embedded. The remainder was immediately immersed in a cold phosphate buffered saline (PBS), quickly cut into small pieces, snap frozen in liquid nitrogen and stored at -70℃ until use. Paraffin sections (6 mm) were stained with hematoxyline and eosin (H&E) for neutrophil evaluation and with toluidine blue for mast cell staining. Neutrophil and mast cell infiltration was evaluated by counting extravascular neutrophils and mast cells in the entire section and averaging the numbers present in 10 serial skin sections from the injection site. Each section was examined independently by three investigators in a blind manner, and the mean was used for analysis.

ELISA for cytokines

Minced samples were weighed and homogenized in 1ml of Passive Lysis Buffer (Promega,

Madison). The supernatants were collected from homogenized samples and used for the measurement. Levels of murine TNF-α (BIOSOURCE, Camarillo, CA), IL-6 (PIERCE ENDOGEN, Rockford, IL) and IL-10 (BIOSOURCE) in the inflammatory site were determined by ELSA kits or immunoassay kits, respectively. The measured values were divided by respective sample weights and calculated values were used for statistical analysis.

Measurement of nitrite

The concentration of nitrite was measured as a stable metabolite reflecting NO by a fluorometric method (NO_2/NO_3 Assay Kit-FX (Fluorometric), 2,3-Diaminonaphthalene Kit, DOJINDO, Kumamoto, Japan). According to the manufacturer's instruction, homogenized solutions were spun at 5000g at 4℃ for one hour using Centricon-10 (Millipore) in order to remove protein. Eighty ml of centrifuged filtrate was applied as samples. Measured values were divided by respective sample weights and calculated values were used for statistical analysis.

Immunohistochemistry for HO-1

The labeled streptavidin biotin (LSAB) method was adopted (DAKO LSAB 2 System, DAKO, Carpinteria, CA), and anti-mouse HO-1 Ab, which was polyclonal (rabbit immunoglobulin), was purchased from StressGen (San Diego, CA). Tissue sections (6 mm) were deparaffinized in xylene. After soaking in 0.05 M Tris-buffered saline (TBS), endogenous peroxidase was blocked with 3% hydrogen peroxide in distilled water for five minutes at room temperature (RT).

After washing in 0.05 M TBS for five minutes, each tissue section was immersed in 0.05 M (TBS) containing 10% normal goat serum for 20 minutes at RT to diminish the non-specific binding of the secondary Ab. Then the sections were treated with anti-mouse HO-1 Ab (1: 250) at 4ºC overnight. The sections were washed and treated with biotinated secondary Ab at RT for 10 minutes and rinsed. Then, the sections were treated with streptavidin-horse raddish peroxidase at RT for 10 minutes. After washing, the slides were treated with substrate-chromogen solution (DAB chromogen) for about 10 minutes at RT. In the end, the slides were counterstained with hematoxylin solution. Negative control staining was obtained by the replacement of the primary Ab with normal rabbit immunoglobulin (DAKO).

Statistical analysis

The Mann-Whitney's U test was used for determining the level of significance of differences in sample means, and Bonferroni's test was used for multiple comparisons. A p value<0.05 was considered statistically significant. All data are shown as means ± SEM.

RESULTS

Edema in the cutaneous reverse Arthus reaction

Edema was assessed by measuring the concentration of Evans blue dye using a spectrophotometer (Fig. 1). Although both positive and negative control mice developed edema significantly 4 and 8 hours after IC formation compared with the levels at 0 hour after IC challenge, edema formation was significantly greater in the positive control mice than in the negative control mice ($P<0.05$). Remarkably, 4 hours after IC challenge, the corrected values of Evans blue dye absorbance in mice treated with hemin, a HO-1 stimulator, were 67% lower than the positive control mice ($P<0.05$) and at a level similar to the negative control mice. By contrast, the mice treated with Znpp-IX, a HO-1 inhibitor, exhibited a significant increase (83%, $P<0.05$) 4 hours after IC challenge. Eight hours after IC challenge, similar results were obtained, although Znpp-IX treatment was not performed. Significantly lower levels of edema were confirmed in the hemin treated mice (52%) and in the negative control mice (68%). Treatment with L-NAME, a NOS inhibitor, did not result in any significant change even at a higher concentration 4 or 8 hours after IC challenge. Thus, hemin treatment inhibited edema, while Znpp-IX treatment augmented it.

Leukocyte infiltration in the cutaneous reverse Arthus reaction

Extravascular neutrophils were assessed in skin tissue sections after 4 and 8 hours of IC formation in the negative control mice, mice treated with 3 mg/kg L-NAME, and mice treated with hemin and compared with the positive control mice (Fig. 2A and 3A). In mice treated with Znpp-IX, skin sections were assessed only after 4 hours of IC formation. Compared with the positive controls, at four hours after IC challenge, neutrophil numbers were significantly lower in the mice treated with hemin (65% lower, $P<0.01$) and near the level of the negative controls, while they were significantly higher in mice treated with Znpp-IX (86% higher, $P<0.05$). In contrast, neutrophil accumulation was not significantly lower in mice treated with 3 mg/kg L-NAME. Similar results were obtained after 8 hours, although Znpp-IX treatment was not performed. Mast cell numbers and localization were also assessed in skin tissue sections stained with toluidine blue (Fig.2B and Fig. 3B). Compared with the positive controls, at four hours after IC challenge, mast cell numbers were significantly lower only in the negative control mice (40% lower, $P<0.05$). Meanwhile, 8 hours after IC challenge, no significant differences in mast cell

number were observed among any of the groups. None of the mice treated with hemin, L-NAME or Znpp-IX exhibited significant changes in mast cell accumulation in the lesional skin. Mast cells were recognized beneath the epidermis, around the perivascular area, in the dermis, in the subcutaneous fat tissue, as well as in and around the muscular area in all groups. On the localization of mast cells, no specific tendencies could be noticed in the present study. Thus, neutrophil infiltration was reduced by hemin treatment, whereas it was augmented by Znpp-IX treatment.

Cytokine levels in the cutaneous reverse Arthus reaction

IC-induced inflammation in the skin is associated with the production and release of proinflammatory cytokines, including TNF-α and IL-6, by infiltrating leukocytes. To assess the degrees of vasculitis in the lesional skin, TNF-α and IL-6 levels were measured in the homogenized samples after 4 and 8 hours of IC formation. Compared with the positive controls, at four hours after IC challenge TNF-α levels in the hemin treated mice ($P<0.05$) were 32% lower and were much closer to the levels of the negative controls. By contrast, Znpp-IX treated mice showed a 44% higher level of TNF-α than the positive controls. Even at the eight hour point similar TNF-α levels were detected in hemin treated and negative control mice ($P<0.05$)(Fig.4).

As for IL-6 (Fig. 5), at 4 hours the hemin treated mice were at a 35% lower level than the positive controls ($P<0.05$), but remained high relative to the negative controls. By contrast, Znpp-IX treated mice showed a 68% higher level of IL-6 ($P<0.01$) compared with the positive controls.

At 8 hours the only significant decrease relative to the positive controls was in the negative control group ($P<0.01$). Thus, hemin treatment displayed significantly lower TNF-α and IL-6 levels, but Znpp-IX treatment displayed significantly higher levels. By contrast, the L-NAME treatments did not show any significant difference in TNF-α or IL-6 levels when compared to the positive controls at either 4 or 8 hours.

IL-10 levels were significantly higher in hemin treated mice compared with the positive controls both 4 and 8 hours after IC challenge (62% and 35% higher, respectively, $P<0.01$) (Fig. 6). Neither L-NAME nor Znpp-IX treatments displayed any significant differences to the positive controls either 4 or 8 hours after IC formation.

Nitrite levels in the cutaneous reverse Arthus reaction

Nitrite levels of the mice treated with 3mg/Kg L-NAME were significantly lower (45%) than those of the positive controls at 4 hours (P<0.05) (Fig. 7). Significantly lower levels of nitrite were recognized in the negative controls (P<0.05) and the mice treated with 3 mg/kg (60%, P<0.01) and 1 mg/kg L-NAME (63%, P<0.01) when compared to the positive controls at 8 hours. Although neither hemin nor Znpp-IX treatments caused any effect, L-NAME treatments actually suppressed nitrite formation in a dose dependent manner.

Immunohistochemical analysis of HO-1 protein

HO-1 expression was reported to be confirmed in keratinocytes, endothelial cells, fibroblasts and macrophages mainly in vitro study [22] [23] [24] [25]. To assess the cell sources of HO-1, cutaneous HO-1 expression during the reverse passive Arthus raction was examined immunohistochemically. In the immunohistochemical examination, HO-1 expression was predominantly expressed in the vesicular cytoplasm of infiltrating macrophages especially shown in the lesional skins of hemin treated mice (Fig. 8). They were mainly scattered in the submuscular area in the lesional skins of hemin treated mice. Thus, HO-1 was predominantly expressed in infiltrating macrophages.

DISCUSSION

Both NO and CO are gaseous molecules. NO is produced by inducible NOS and CO by HO-1, especially in the lesions of inflammation, because they are both induced types. We selected hemin as a chemical HO-1 inducer, Znpp-IX as a competitive HO-1 inhibitor and L-NAME as a NOS inhibitor in the present study. The levels of TNF-α, IL-6, Evans blue dye and the numbers of infiltrating neutrophils at the lesions were significantly higher in the positive control mice than those in the negative control mice both 4 and 8 hours after IC formation. Therefore, these four parameters were thought to be good markers for the evaluation of this cutaneous reverse Arthus reaction. The increase in the number of neutrophils and masct cells in the negative control may be due to the weak inflammatory response against rabbit IgG Ab. A zero control group showed a relatively high amount of TNF-α and low concentration of IL-6, although these were both proinflammatory cytokines. They have been reported to be produced and released by cells such as neutrophils, monocytes and mast cells [26] [27]. It was also reported that a low expression level of IL-6 was recognized, although an increase of TNF-α was very prominent at 1 hour after stimulation in the in vitro experiment using mouse monocytes [28]. Therefore, it seemed to be possible that the response of TNF-α was more prompt than that of IL-6 during the very early stage of inflammation, although crucial stimuli could be uncertain in the present study.

Meanwhile, a significant decrease in the value of IL-6 was not recognized at 8 hours among 4 parameters, although the decreases in the other 4 parameters were significant both at 4 and 8 hours after IC formation in hemin treated mice when compared to positive control mice. HO-1 has been reported to suppress TNF-α, IL-6 and IL-8 [29]. Although we did not examine IL-8 levels, we did confirm a significant decrease in the number of neutrophils infiltrating into inflammatory sites in the hemin treated mice. Actually, we did not confirm a significant decrease in the value of IL-6 at 8 hours, but we did recognize a significant decrease in the value of IL-6 at 4 hours after IC formation in hemin treated mice when compared to positive control mice. Therefore, we believe that our results support their findings. Some papers reported the involvement of TNF-α in the pathogenesis of edema formation [30]. Our results made us consider the possibility that IL-6 was not mainly involved in the pathogenesis of edema formation 8 hours after IC formation in the present study. In order to confirm whether or not this improvement was caused via HO-1/CO signalling pathway, we additionally conducted Znpp-IX treatment,

which was a competitive HO-1 inhibitor at 4 hours in the present study. As expected, quite the reverse reactions were observed in Znpp-IX treated mice 4 hours after IC challenge. These results indicate that the HO-1/CO signalling pathway could be involved in the improvement of the cutaneous reverse Arthus reaction. Inflammatory sites contained edematous stromas where many cells were recruited, including macrophages. We confirmed HO-1 expression immunohistologically in the cytoplasm of macrophages infiltrating in the inflammatory sites especially in hemin treated mice. Since previous studies have shown that the cell source of HO-1 was macrophages [31], it was recognized that the supply of HO-1 was thought to be mainly derived from macrophages in the stromas of inflammatory sites. Therefore, it could be recognized that the improvement of vasculitis in hemin treated mice and the exacerbation in Znpp-IX treated mice depended on HO-1/CO signalling pathway. Actually, we injected rabbit IgG antibodies intradermally with a 29-gauge needle, according to the original method. However, we were not able to confirm capillary vessels damaged beneath the epidermis like an anaphylactoid purpura, although we have reported some papers using this model〔3〕〔4〕. Namely, damaged vessels usually exist deep around muscular layer and subcutaneous fat tissue in this model (Fig.2A). We thought that the levels of damaged vessels depended on the types of injected immunoglobulin. Therefore, we tried the intradermal injection of IgA antibodies, which were confirmed in the lesional skins of anaphylactoid purpura, in this model, but we could not recognize damaged vessels even in the superficial dermis. (Data not shown) In the end, we cannot explain the discrepancy in this model. It is however the fact that diseased vessels exist deep around muscular layer and subcutaneous fat tissue, although we actually injected rabbit IgG antibodies intradermally in the cutaneous passive reverse Athus reaction. We now consider that diseased vessels exist deep around muscular layer and subcutaneous fat tissue in this model, so macrophage infiltration is mainly located in deep subcutaneous area as we showed in Fig. 8.

Meanwhile, L-NAME was selected as a NOS inhibitor in the present study. Although the actual decrease of nitrite in the inflammatory lesions, which was one of the stable metabolites of NO, was confirmed in a dose dependent manner, neither significant improvement nor exacerbation was recognized in mice treated with 1 mg/kg and 3 mg/kg L-NAME, at both 4 and 8 hours after IC formation. Some reports have mentioned that NO acts as a cytodestructive molecule in some pathological conditions [32], but the inhibition of NOS did not show any improvement in the cutaneous reverse passive Arthus

reaction. Namely, none of four markers of vasculitis showed any improvement in the mice treated with L-NAME. Although CO was reported to selectively suppress the expression of inducible NOS [33], hemin treatment, which showed remarkable improvements of four markers of vasculitis, did not suppress lesional nitrite. Therefore, it can be considered that for the improvement of this cutaneous reverse Arthus reaction, at requisite is an increase of HO-1 but not a decrease of nitrite. Therefore, it was speculated that hemin did improve vasculitis independent of NOS/ NO pathway.

We confirmed significant increases of IL-10 in mice treated with hemin both 4 and 8 hours after IC formation. It has been reported that IL-10 was an anti-inflammatory cytokine and HO-1 could induce IL-10 [29, 34]. Thus, the possibility was recognized that HO-1 could improve inflammation via IL-10 signaling pathway in the cutaneous reverse passive Arthus reaction. However, the suppression of IL-10 was not observed in mice treated with Znpp-IX, which was a competitive inhibitor of HO-1. We also confirmed that the zero control group contained a relatively high amount of IL-10, which was reported to be produced by some cells including monocytes, keratinocytes and mast cells［35］. It was reported that IL-10 was constitutively expressed and secreted by the human normal colonic mucosa［36］. Furthermore, TNF-α can also induce IL-10 because of negative feedback mechanism. Therefore, a relatively high amount of IL-10 was detected during the very early stage of cutaneous inflammation in the present study.

Mast cell recruitment into tissue is thought to occur by release of immature mast cell precursors from the bone marrow into the peripheral blood, followed by migration of these precursors into tissues and their subsequent differentiation into mature mast cells [37]. Increased numbers of mast cells are noticed at the sites of inflammation [38]. Indeed, we confirmed a significant increase of mast cell number in the positive control mice compared with the negative controls only 4 hours after IC formation, and no significant differences were seen between the L-NAME treated, hemin treated, positive control and negative control mice 8 hours after IC challenge. As L-NAME, hemin and Znpp-IX treatment did not affect mast cell numbers, it was considered that the improvement or exacerbation of vasculitis occurred without relation to mast cell function in the present study. Further experiments should be conducted in order to clarify the exact role of mast cells in the inflammation associated with the Arthus reaction.

In conclusion, the results of this study indicate that the HO-1/CO signalling pathway plays a critical role in the improvement of the cutaneous passive reverse Arthus reaction.

Furthermore, our results may provide important basic information for the therapy of IC-mediated human diseases by the development of drug that can increase HO-1/CO signalling pathway.

LEGENDS

Fig. 1

Edema in the cutaneous reverse passive Arthus reaction. Mice were injected intradermally with rabbit IgG anti-chicken egg albumin Ab, followed by intravenous injection of chicken egg albumin and 0.4% Evans blue dye served as positive controls. Dorsal skins were harvested form mice 0, 4 and 8 hours after IC challenge. Mice that received an intradermal injection of polyclonal rabbit IgG followed by intravenous installation of chicken egg albumin served as negative controls. The zero control group meant a positive control group sacrificed immediately after positive IC challenge. L-NAME was adopted as a NOS inhibitor, hemin as a chemical HO-1 stimulator and Znpp-IX as a competitive HO-1 inhibitor. The absorbances of samples and standard series were measured at 620 nm using a spectrophotometer, and the concentration of Evans blue dye in each sample was calculated against the calibration curve of the standard Evans blue solution.

Fig. 2

Histological tissue sections showing neutrophil infiltration (A) and mast cell accumulation (B) in the lesional skin at 4 and 8 hours after IC challenge. Neutrophils were revealed by H&E staining and mast cell (arrow) were detected as cells with metachromatic staining of granules in toluidine blue-staining. Original magnifications were x 100 (A) and x 50 (B), respectively. With regard to Znpp-IX, positive control, L-NAME and hemin, refer to the legends in Fig. 1.

Fig. 3

Arthus reaction-induced recruitment of neutrophils (A) and mast cells (B) in the lesional skins. Numbers of neutrophils and mast cells per skin section were determined by counting in H&E- and toluidine blue-stained skin sections. All values represent the mean ± SEM of results obtained from 5 to 10 mice in each group. Statistical analysis is provided in the Results. With regard to Znpp-IX, positive control, L-NAME, hemin and negative control, refer to the legends in Fig. 1.

Fig. 4

Arthus reaction-induced TNF-α production in the homogenized, lesional skin from each group at 0, 4 and 8 hours after IC challenge. TNF-α levels in the lesional skins were determined by ELISA. Measured values were corrected by respective sample weights.

All corrected values represent the mean ± SEM of results obtained from 5 to 10 mice in each group.

＊, P<0.05 and ＊＊, P<0.01 versus levels of positive control group at 4 and 8 hours after IC challenge.

Figure 5

Arthus reaction-induced IL-6 production in the homogenized, lesional skin from each group at 0, 4 and 8 hours after IC challenge. IL-6 levels in the lesional skins were determined by ELISA. Measured values were corrected by respective sample weights. All corrected values represent the mean ± SEM of results obtained from 5 to 10 mice in each group.

＊, P<0.05 and ＊＊, P<0.01 versus levels of positive control group at 4 and 8 hours after IC challenge.

Fig. 6

IL-10 production in the homogenized, lesional skin from each group at 0, 4 and 8 hours after IC challenge. IL-10 levels in the lesional skins were determined by ELISA. Measured values were corrected by respective sample weights. All corrected values represent the mean ± SEM of results obtained from 5 to 10 mice in each group. ＊, P<0.05 and ＊＊, P<0.01 versus levels of positive control group at 4 and 8 hours after IC challenge.

Fig. 7

Nitrite was measured as a stable metabolite reflecting NO in the homogenized samples. Measured values were corrected by respective sample weights. All corrected values represent the mean ± SEM of results obtained from 5 to 10 mice in each group. ＊, P<0.05 and ＊＊, P<0.01 versus levels of positive control group at 4 and 8 hours after IC challenge.

Fig. 8

Histological tissue sections showing macrophage infiltration in the skin of hemin treated mice at four hours after IC challenge.

(A): Sections were stained with H&E staining. Original magnification, x25. High magnification of infiltrating macrophage, the cytoplasm of which was vesicular. (x100, insert)

(B): HO-1 expression in skin from hemin treated mice during cutaneous passive Arthus reaction. HO-1 expression in the lesional skin from hemin treated mice after 4 hours of IC

challenge was assessed by immunohistochemistry using anti-HO-1 Ab. Sections were counterstained with hematoxyline. Original magnification, x25, x50 (insert).

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