Skin-organ culture as an approach in evaluation of nitric oxide (NO) involvement in contact hypersensitivity expression

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ABSTRACT

Objective. Contact hypersensitivity (CHS) is a local inflammatory response of the skin following challenge of hapten-sensitized animals. The intensity of inflammation could be quantified by ear swelling, a classical manifestation of contact hypersensitivity. In this study, skin-organ culture system was employed to evaluate involvement of nitric oxide (NO) in the expression of CHS in rats.

Methods. Nitrite accumulation in conditioned medium (CM) of rat ear skin following challenge with DNCB was determined by Griess assay. The experiment was conducted on two genetically different rat strains (AO and DA), which differ in the magnitude of ear swelling response.

Results. Dose-dependent increase in nitrite levels was noted in CM following application of 0.65% and 1.3% DNCB to the ears of sensitized rats of both strains. The response was higher in DA rats, displaying a more vigorous ear swelling. Correlation between ear swelling and the levels of nitrites in CM of both strains was found. Decrease in nitrite levels was noted in CM in the presence of aminoguanidine, a known inhibitor with marked specificity for the inducible isoform of the NO synthase in rodents.

Conclusion. Presented data demonstrated the utility of skin organ-culture system in detection of NO production during the expression of CHS.

KEY WORDS

contact hypersensitivity, skin organ culture, nitrites

Introduction

Contact hypersensitivity (CHS) is a T cell-mediated inflammatory response of the epidermis following skin challenge of hapten-sensitized animals. The afferent phase of contact hypersensitivity response, sensitization phase, is initiated by epicutaneous application of the hapten to dorsal or abdominal trunk skin, and is characterised by the activation and division of hapten-specific T lymphocytes in the regional lymph nodes and the appearance of effector cells in the lymph nodes and spleen (1). In the efferent phase, which follows a subsequent challenge with hapten, e.g. epicutaneous application of the sensitizer to the skin of the ear, primed T lymphocytes are recruited to the site of challenge where they produce a variety of inflammatory mediators, amplifying a background inflammatory response into a more vigorous process. This is the classical manifestation of contact hypersensitivity that is measured as "ear swelling". Ear swelling is the early-recognized skin

response to hapten application, characterized histologically by dermal cell infiltration (2). The magnitude of ear swelling reflects the intensity of local inflammatory response determined largely by the presence and activity of cytokines (3,4) and adhesion molecules (5,6), which govern leukocyte extravasation and effector functions *in situ*. Among cytokines, tumor necrosis factor-α (TNF-α) seems to play a major role in elicitation of CHS, as demonstrated in the mouse model of CHS to trinitrochlorobenzene (TNCB) (3).

Recent data demonstrated the involvement of nitric oxide (NO) in the expression of CHS to dinitrofluorobenzene (DNFB) in mice (7). Contribution of NO synthesis was indicated in this model of CHS and the activity of inducible nitric oxide synthase (iNOS) has been suggested as largely responsible for NO production during CHS (7). These data are in line with the reported expression of inducible nitric oxide synthase (iNOS) in various types of skin cells, and with the postulated role of synthesis of this biological mediator in inflammatory/immune-mediated processes in skin (8).

Short-term culture of skin explants is a new experimental *in vitro* system designed to emulate *in vivo* conditions. Development of skin organ culture techniques has provided investigation of various aspects of normal and pathological skin biology including maintenance of homeostasis, inflammation, effects of chemical and physical agents, as well as cell migration (9). Cutaneous inflammation has been investigated frequently by monitoring the presence of various soluble biochemical and inflammatory/immunoregulatory mediators released from skin explants in culture fluids. In this way, various mediators are being collected and defined as relevant for cutaneous inflammation including biologically active amines, proteolytic enzymes, various serum proteins and cytokines (10,11,12).

Using a rat model of experimentally induced contact hypersensitivity reaction to dinitrochlorobenzene (DNCB) (13,30) we have demonstrated a rise in levels of TNF-α in conditioned medium of organ-cultured ear skin following challenge with increased doses of DNCB (9). Since correlation was noted between the magnitude of ear swelling response and an increase in levels of TNF-α in conditioned medium of organ-cultured ear skin following challenge, the utility of this experimental system in evaluation of inflammatory mediators involvement in CHS expression was suggested (9). In this study, organ culture of rat skin was used as an approach in evaluation of nitric oxide involvement in expression of contact hypersensitivity to DNCB in rats. To this aim, accumulation of nitrites was determined in culture fluids of organ-cultured ear skin following challenge, concomitantly with changes in ear thickness as a classical measure of CHS expression. As there are strain differences in reactivity to contact sensitization in rats (14), experiments were conducted in two genetically different rat strains Albino Oxford (AO) and Dark August (DA), which differ in the magnitude of ear swelling response to DNCB (15).

Materials and methods

Chemicals and reagents

1-chloro-2,4-dinitrochlorobenzene, DNCB (BDH Chemicals LTD, England) was dissolved in vehicle (acetone and olive oil, 4:1). AminoguAnidine (Sigma, USA) was dissolved in culture medium RPMI-1640 (ICN Flow, USA) supplemented with foetal calf serum, FCS 5% (v/v) and gentamycin (1ml /L).

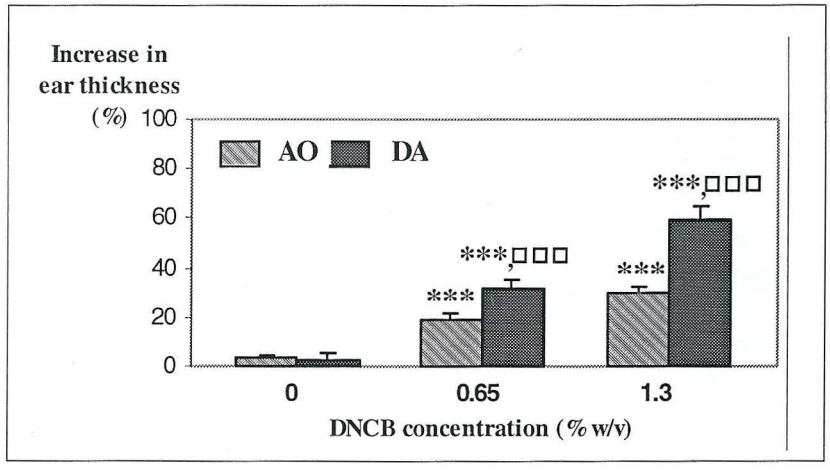
Animals

All experiments were done in adherence to the NIH guidelines for the use of experimental animals, with permission of the Ethical Committee of our Institute. Inbred male Albino Oxford (AO) and Dark August (DA) rats (Farm for Experimental Animals, Military Medical Academy, Belgrade, Yugoslavia), 3 months of age, were housed in an air-conditioned rooms at 25°C on a 12-h light/dark cycle. Animals were provided pelleted food and water *ad libitum*. During the experiment, animals were caged individually.

Contact sensitization and ear swelling response

Groups of six to eight animals received 100 µl of DNCB (2% and 4%, w/v in vehicle) or an equal volume of vehicle (4:1 acetone: olive oil) on the shaved area for two consecutive days. Three days later, rats were chal-

Fig 1. Mean percent increase in ear thickness following challenge of AO and DA rats. Results are expressed as means ± SD for each animal group (7 animals). Significance at * p<0.05, **p<0.01 or ***p<0.001 *vs* vehicle (0% DNCB) treatment or p<0.001 *vs* respective values for AO rats.



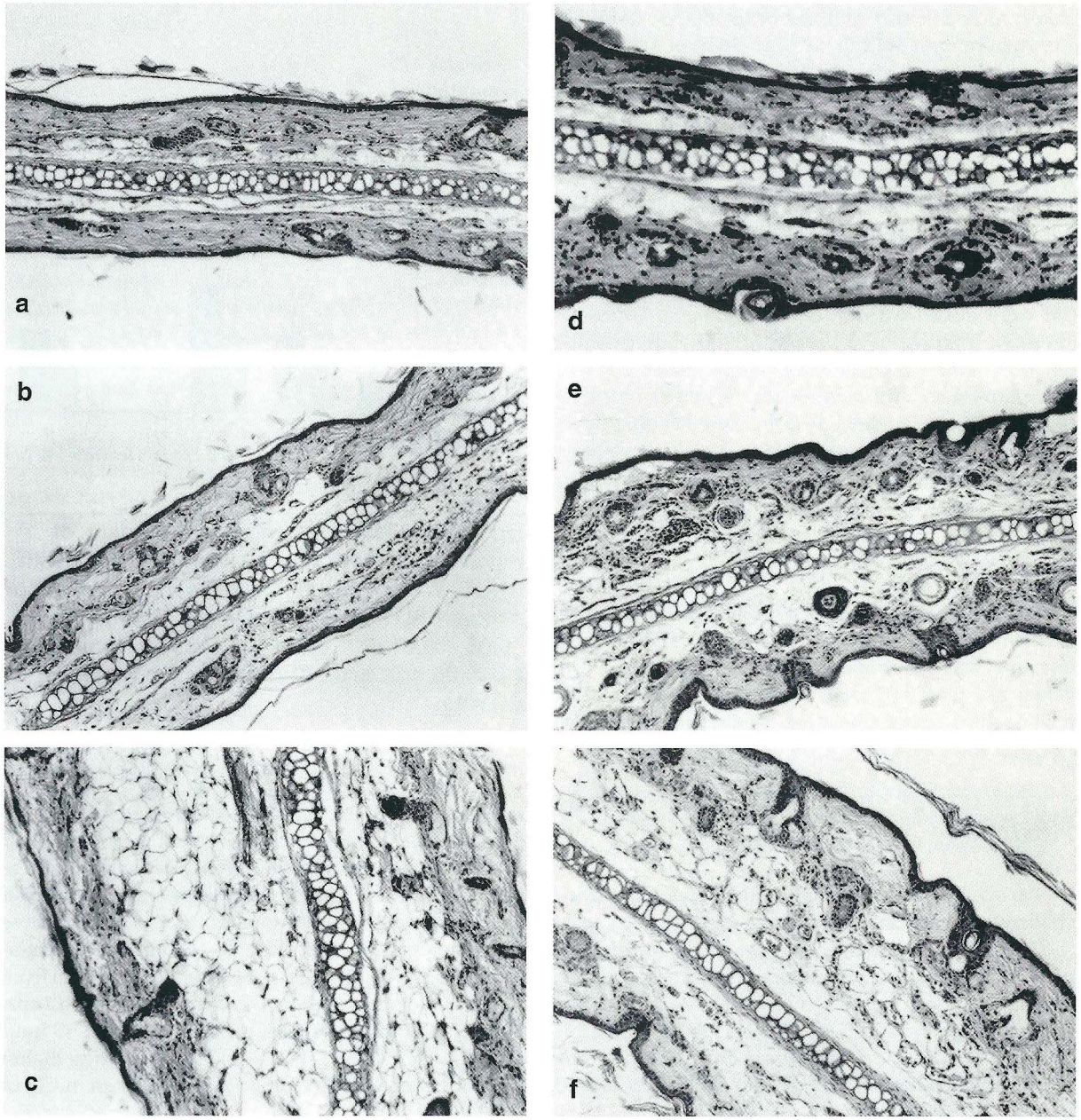


Fig 2. Histology of ear skin from AO (A-C) or DA (D-F) rats following application of 0.66% (B, E) or 1.3% (C, F) of DNCB or following application of vehicle solely (A, D). H&E, x 32.

lenged by application of 50 µl of three times lower concentration of DNCB than that used in sensitizations phase (i.e. 0.66% and 1.3%) on the dorsal skin of the left ear. The contact hypersensitivity reaction was assessed by measuring the ear thickness with an engineer's micrometer 24 hours after challenge. The response was quantitated as the difference in the thickness between challenged and non-treated ears of the same animal, according to the formula (C-N)/Nx100, where C is thickness of the challenged ear, N is thickness of non-treated ear. The response was expressed as percent of increase in ear thickness.

Histology

Ear samples taken 24 h after challenge were placed into 4% buffered formalin, pH 6.9, embedded in paraffin wax for sectioning at 5 µm and stained with hematoxylin/eosin (H&E).

Skin Organ Culture

Exposed ears were taken 24 h after elicitation of the CHS, split into ventral and dorsal halves and submerged in 1.5 ml of culture medium in wells of 24-well culture

plates. After 24h and 48 hours of incubation in humid atmosphere with 5% CO_2 at 37°C (Heräus, Germany), the conditioned medium (CM) was collected. In some experiments, ear halves were cultured in the presence of 25 and 50 μg/ml aminoguanidine.

Nitrite measurement

Nitrite accumulation, as indicator of NO production, was measured in 24 h and 48 h conditioned medium using the Griess reagent (16). Briefly, 50 μl aliquots of CM were mixed with an equal volume of Griess reagent (a mixture of 0.1% naphtylethylenediamine dihydrochloride in water and 1% sulphanilamide in 5% phosphoric acid) and incubated for 10 min at room temperature. The absorbance was measured at dual wavelength 570nm/650nm by an ELISA 96-well plate reader (Behring, FR Germany). The nitrite concentration was calculated from a sodium nitrite standard curve.

Statistical analysis

Results are expressed as mean value ±SD for each experimental animal group (6-8 animals). Statistical significance was determined by the Mann-Whitney test. P values less than 0.05 were considered significant. The relationship between the levels of nitrites in CM and ear swelling was examined by regression analysis.

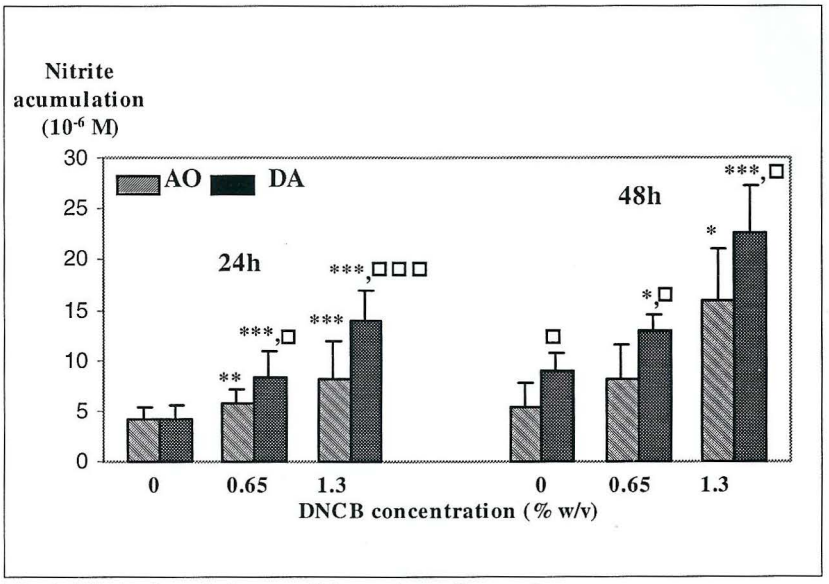


Fig 3. Nitrite levels in conditioned medium of organ-cultured ear skin following challenge with DNCB. Values are expressed as means of mM of nitrites.± SD from seven animals. Significance at * 0.05, **0.01 or ***0.001 *vs* vehicle treatment or ‡ p<0.05, ‡‡‡ p<0.001 *vs* respective values for AO rats.

Results

Ear swelling response and skin histology following challenge of ears of sensitized animals

The mean percentage increase in ear thickness is presented in Fig. 1. A dose-dependent increase in ear swelling response was evident for both AO and DA rats, being more pronounced in animals of DA strain. Application of DNCB to the ears of sensitized animals produced both epidermal and dermal alterations characteristic for contact dermatitis. Histological picture of ear skin 24 hours after challenge of AO and DA rats with DNCB is presented in Fig. 2 illustrating the increase in ear oedema following application of hapten, compared to vehicle. The higher the dose of the hapten, more intense skin oedema was noted compared to vehicle application. In the dermis, oedema was associated with leukocyte infiltration: 14±2 and 29±8 cells/microscopic field in skin of AO rats and 19±4 and 34±4 cells/microscopic field in skin of DA rats following challenge with 0.65% and 1.3% DNCB, respectively, compared to 9-10 cells/field in the dermis of vehicle treated rats.

Nitrite levels in culture fluids of ear skin explants

Nitrite accumulation was measured in conditioned medium (CM) of ear skin from challenged AO and DA rats (Fig 3). Increased levels of nitrites were evident in 24-hour conditioned medium (CM) following application of 0.65% and 1.3% DNCB to the ears of both sensitized AO and DA rats compared to CM of respective control, vehicle treated ears. Rise in nitrite concentrations was greater in CM of organ-cultured ear skin from DA rats compared to these measured in CM of AO rats. Further increase in nitrite levels was noted in 48-hour skin-conditioned medium of both strains, being higher in CM of skin from DA rats and evident even in CM of vehicle-treated ears.

There is a correlation between increase in ear thickness and levels of nitrites in 24-hour and 48-hour CM of ear skin from AO rats (Fig 4 A, B) (the respective formula is y=2.836x - 1.101, r=0.76, p<0.001 for 24-hour CM and y=1.6188x+ 1.3466, r=0.785, p<0.001for 48-hour CM. Significant correlation was noted between respective parameters in DA rats (Fig 4 C, D). The respective formula is y=4.5884x-6.6693, with r =0.828 and p<0.001 for 24-h CM and y= 2.2527x, r=0.866, p<0,001, for 48-hour CM.

Effect of aminoguanidine on nitrite accumulation.

In order to examine contribution of NO production to the observed increase in nitrite accumulation in cul-

ture fluids of ear skin, specimens harvested following challenge with DNCB or following vehicle application to DA rats were cultured in the presence of aminoguanidine, inducible isoform of nitric oxide synthase (iNOS) inhibitor, with a marked specificity for iNOS in rodents (17). As shown in Fig 5, significant and dose-dependent reductions in nitrite levels were noted both in 24-hour and 48-hour CM of ear skin explants cultured with 25 μg/ml and 50 μg/ml of aminoguanidine.

Discussion

Contact hypersensitivity (CHS) is a local inflammatory response of the skin following challenge of sensitized animals. The intensity of inflammation following hapten application could be quantified by ear swelling, since dose-dependent increase in ear thickness was noted. Ear swelling is the early recognized manifestation of contact hypersensitivity characterised by dermal cell infiltration (2), identified as T cells, granulocytes, monocytes /macrophages, and Langerhans cells (18). It is based largely on activity of skin cells, which through release of cytokines modulate skin microenvironment facilitating extravasation of inflammatory leukocytes. In the dermis, leukocytes produce a variety of inflammatory mediators, amplifying the local response into vigorous inflammatory process expressed as ear swelling (19). This local inflammatory response is determined by protein-type factors (cytokines), (3,4) adhesion molecules (5,6) and, as recently demonstrated, nitric oxide (7).

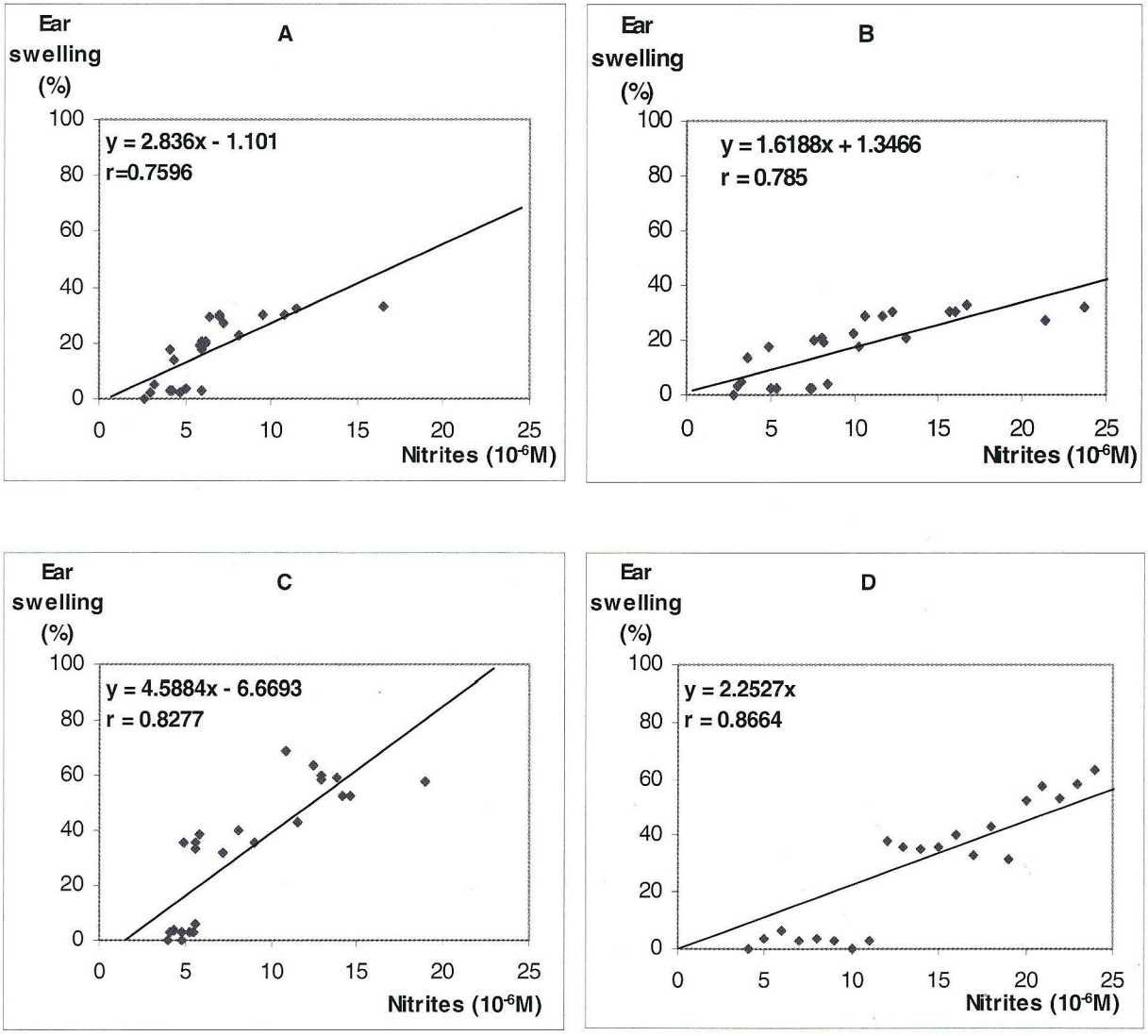


Fig 4. The regression lines for changes in ear swelling and nitrite levels in conditioned medium of ear skin following challenge of AO (A, B) or DA (C, D) rats.
24 hour-conditioned medium (A, C) or 48 hour-conditioned medium (B, D).

In this study, the expression of contact hypersensitivity was evaluated by traditional measurements of changes in ear thickness following challenge with DNCB and by organ-cultured skin approach. Nitrite concentrations were determined using organ-culture in culture fluids of ears following hapten challenge. The measure of NO production was estimated with the Griess reaction, since it is only possible to evaluate the concentration of NO indirectly, measuring its metabolites - nitrites/nitrates (20). In concordance with data, which demonstrated measurement of nitrite accumulation in culture supernatants of macrophages as an indicator of NO production in conditions of inflammation (21, 22), our data demonstrated the utility of the ear skin organ culture system in nitrite level measurements in conditioned medium of tissue (skin) explants. Our results that demonstrated dose-dependent increase in ear thickness following administration of DNCB (noted both in AO and DA rats), which was accompanied by increase in levels of nitrites in challenged ear-skin conditioned medium, suggest the utility of nitrite accumulation measurements in conditioned media as an approach in evaluation of NO contribution to CHS expression. Data demonstrating higher levels of NO_2^- in CM of ear skin from DA rats, which respond to DCNB challenge with more vigorous inflammatory response (greater magnitude of ear swelling), support this assumption.

Significant correlation between levels of nitrites in CM of skin following DNCB challenge and the magnitude of ear swelling response suggested relationship between nitrite accumulation in skin culture fluid and the intensity of local skin inflammatory response during the elicitation of CHS. These data are further in line with that obtained in mouse model of CHS to DNFB which demonstrated involvement of nitric oxide in ear swelling response and contribution of NO production to this response (7).

The skin organ culture system has been exploited in detection of various inflammatory mediators produced and released by skin explants in culture fluid (10,11,12,23,9). It appeared to be valuable in studying local skin cytokine response following topical application/exposure to various chemical and physical agents including heat, mechanical injury, some drugs and toxicants (12,23). Using this experimental system a rise in levels of TNF-α in conditioned medium of organ-cultured ear skin explants was noted following application of increased doses of DNCB to the ears of sensitized rats (9). As correlation was established between the intensity of ear swelling response to DNCB and an increase in levels of TNF-α in conditioned medium of organ-cultured ear skin following challenge, the utility of this experimental system in evaluation of inflammatory mediators involvement in CHS expression was suggested (9). Presented data suggest nitrite accumulation measurements in challenged ear skin conditioned media as an approach in evaluation of NO contribution to CHS expression.

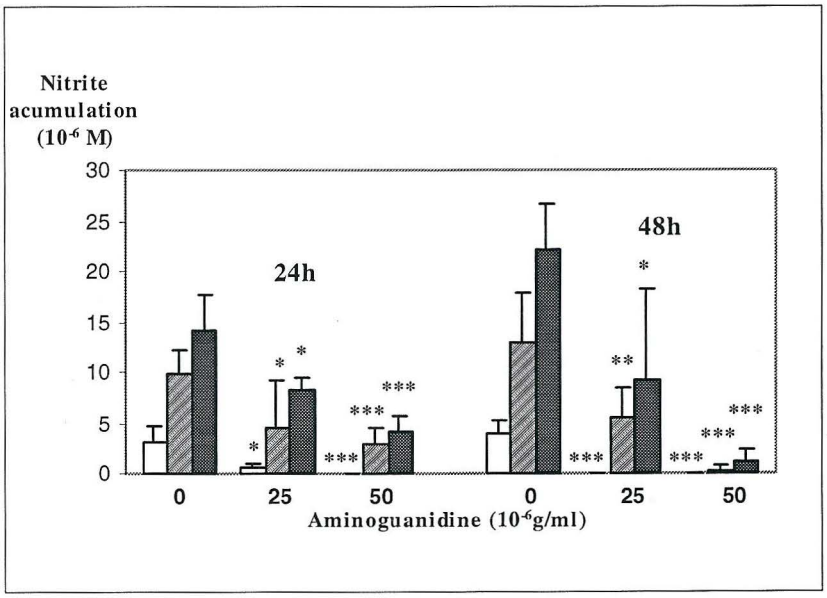


Fig. 5. Nitrite levels in conditioned medium of ear skin from DA rats, cultured in the presence of aminoguanidine. Values expressed as means of mM of nitrites.± SD from seven animals. Significance at * p<0.05, **p<0.01 or ***p<0.001 *vs* nitrites in CM from cultures without aminoguanidine.

Our data that demonstrated a drop in nitrite levels in CM in the presence of aminoguanidine, which has a marked specificity for iNOS in rodents (17), suggested that NO production could be modulated in skin-organ culture. The results of these inhibition experiments corroborate data obtained in mice *in vivo* in which inducible NOS is suggested as largely responsible for NO production during CHS to DNFB in mouse. In that model of CHS, keratinocytes and Langerhans cells were suggested to be NO producing cells (7). NO produced by these cells may be involved in CHS reaction by formation of oedema and inflammatory cell infiltrate during effector phase. We suggest that leukocyte (cells in dermal infiltrate) NO production, also contribute to skin NO production. The dynamics of nitrite accumulation, with highest values obtained by 48 hours in culture, are in concordance with that of rat leukocytes (macrophages and granulocytes) in culture (22 and unpublished data). Inflammatory cells stimulated by epidermal cell cytokines as well as their own cytokines produce NO and thereby amplification could occur. From numerous studies it is known that inflammatory cytokines (IFN-γ, IL-8, TNF-α, IL-1 and others) could stimulate keratinocytes to produce NO (8) and that some (e.g. IFN-γ) have a capacity of stimulating iNOS expression in Langerhans cells (24). In addition, TNF-α (21) and IFN-γ (25) could stimulate macrophage NO production. Following hapten application, skin cell-derived NO could induce vasodilata-

tion, causing an increase in the volume of blood and delivery of leukocytes at the site of inflammation. The role of NO in formation of inflammatory cell infiltrate in dermis was supported by recent data which demonstrated the relevance of nitric oxide in neutrophil recruitment to inflammatory focus in various models of inflammation (26). Inhibitors of NO synthesis employed in these models of inflammation have caused reduction in the accumulation of neutrophils at the site of inflammation (26).

One aspect regarding nitrite measurements in culture fluids of ear skin should be mentioned. Skin organ culture has been recommended recently as a new experimental system suitable for studying and testing the effects of various physical, chemical and biological agents on skin (27). As CHS is frequently employed in investigations of various agents of immunotoxicity (28, 29), determination of changes in nitrite levels, as indicators of NO production in skin-organ culture system might be used as an accompanying test. Thus valuable information regarding agents causing skin toxicity and inflammatory cell accumulation/function in contact hypersensitivity could be obtained.

Conclusion

In conclusion, data presented in this paper demonstrate the evaluation of CHS response by *in vitro* measurement of nitrite accumulation in culture fluids of organ-cultured skin. The relevance of measurement of nitrites as an indicator of NO production by inflamed skin in CHS is indicated by a correlation between ear swelling and levels of nitrite accumulation.

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