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Intracellular adhesion molecule-1 up-regulation on thyrocytes by iodine of non-obese diabetic.H2^{h4} mice is reactive oxygen species-dependent

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Summary

Intracellular adhesion molecule-1 (ICAM-1) expression on the thyroid follicular cells of non-obese diabetic (NOD).H2^{h4} mice is enhanced by iodide treatment, which correlates with autoimmune thyroid disease in genetically susceptible NOD.H2^{h4} mice. The current study examines the mechanism of iodine-enhanced up-regulation of ICAM-1 on the surface of thyroid cells. We hypothesized that the up-regulation of ICAM-1 is due to a transient increase in production of reactive oxygen species (ROS). ROS may initiate signalling of the ICAM-1 gene promoter, enhancing up-regulated ICAM-1 protein on the cell surface. Single-cell suspensions of thyroid follicular cells from thyroiditis-susceptible NOD.H2^{h4} or non-susceptible BALB/c mice were treated *in vitro* with sodium iodide. Extracellular and intracellular ROS were assessed by luminol-derived chemiluminescence and flow cytometry assays respectively. Our results demonstrate that thyroid follicular cells of NOD.H2^{h4} generate higher levels of ROS compared with cells from non-susceptible strains of mice. Expression of a subunit protein of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, p67^{phox}, was analysed by Western blot immunoassay. A constitutive expression of the p67^{phox} subunit protein was observed in NOD.H2^{h4} mice prior to iodine treatment. No such expression was found in BALB/c mice. Treatment of NOD.H2^{h4} thyroid cells with diphenyleneiodium, an inhibitor of NADPH oxidase, reduced generation of ROS and of ICAM-1 protein expression. Thus, thyrocytes from NOD.H2^{h4} mice produce enhanced levels of ROS that may be mediated by NADPH oxidase. Consequently, in NOD.H2^{h4} mice the ROS-induced signal for ICAM-1 up-regulation may contribute to mononuclear cellular infiltration of the thyroid gland and the progression of autoimmune thyroid disease.

Keywords: adhesion molecules, autoimmunity, gene regulation, rodent

Introduction

The non-obese diabetic (NOD).H2^{h4} mouse, expressing the major histocompatibility complex haplotype H-2^{k} on a NOD background, is a well-established model of spontaneous autoimmune thyroiditis (AT) [1–3]. The H-2^{k} haplotype is associated with a strong immune response to thyroglobulin, a major antigen in AT [4]. Progressive infiltration of mononuclear cells into the thyroid gland and thyroid-specific antibody production is accelerated in NOD.H2^{h4} mice upon dietary supplementation with iodine [2,3,5]. Supplementation of iodine to non-susceptible mice such as BALB/c or B10.A mice does not enhance AT, affirming a genetic predilection to autoimmune disease because of the NOD background [6]. The NOD.H2^{h4} mouse therefore provides an excellent model to study the mechanisms of iodine-enhanced autoimmune thyroid disease.

We have reported previously that NOD.H2^{h4} mice express intracellular adhesion molecule-1 (ICAM-1) on their thyrocytes constitutively, and that iodine supplementation in their drinking water up-regulated this ICAM-1 expression [6,7]. *In vitro* iodine treatment of isolated thyroid follicular cells also demonstrated enhanced expression of ICAM-1 on their surface [6]. Because ICAM-1 has been shown to play a major role in the early stages of inflammatory responses, we predicted that this up-regulation of ICAM-1 on thyrocytes could be one mechanism to explain the accelerated

mononuclear cell infiltration into the thyroid glands during the autoimmune process.

The primary goal of this study, therefore, was to examine the mechanism of ICAM-1 expression on the thyroid follicular cells of NOD.H2^{h4} mice in response to iodine. Iodine is required for the normal functioning of the thyroid gland in humans as well as in all other animals, including mice. It is taken up primarily by thyroid follicular cells and used for the synthesis of thyroid hormones. Reactive oxygen species (ROS) are involved in the biochemical synthesis of thyroid hormones; iodides are oxidized by the enzyme thyroid peroxidase using hydrogen peroxide (H_2O_2) to form iodinated tyrosyls [8,9]. ROS, as well as many other stimuli, such as cytokines, viruses, bacterial lipopolysaccharide, radiation and retinoic acid, are known to stimulate the ICAM-1 gene promoter [10–12]. In genetically susceptible individuals AT may well be triggered through these diverse stimuli, so that ICAM-1 is over-expressed on the thyroid follicular cells. ICAM-1 has been demonstrated on the thyrocytes of patients with autoimmune thyroid disease both *in vivo* and *in vitro* [13–16]. However, whether this increased expression of ICAM-1 was a cause or result of cellular infiltration is not known. To understand if iodine could directly enhance ROS generation that would account for up-regulated ICAM-1 and increased susceptibility to AT, we performed experiments with thyrocytes isolated from NOD.H2^{h4} mice and compared them with cells from non-susceptible strains of mice.

To pursue this hypothesis we investigated if iodine directly enhances ICAM-1 surface protein on thyroid cells, that is associated with the generation of ROS. Furthermore, using the pharmacological inhibitor diphenyleneiodium (DPI), we examined the role of ROS in ICAM-1-mediated disease pathology in susceptible NOD.H2^{h4} mice.

Materials and methods

Mice

Non-obese diabetic.H2^{h4} mice were born and reared at the Johns Hopkins School of Medicine specific pathogen-free housing animal facility. Additionally, B10.A and BALB/c and NOD.H2^{h4} mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The original NOD.H2^{h4} strain was developed by Dr Linda Wicker (Merck Pharmaceutical Laboratories, Rahway, NJ, USA) by crossing NOD mice with B10.A (4R) followed by back-crossing to the parental NOD [1]. Thus, the NOD.H2^{h4} mouse expresses the thyroiditis-susceptible I-A^k allele on the NOD background [4]. Both males and females develop thyroiditis in this strain [3,5]. Each experimental group consisted of pooled thyroid cells from B10.A, BALB/c or NOD.H2^{h4} mice ($n = 7$–10) approximately 8–10 weeks of age and from both sexes. NOD.H2^{h4} mice transgenic for interferon (IFN)-γ in their thyroid gland were derived from founder mice developed by Dr Patrizio Caturegli, Department of Pathology, Johns Hopkins University [17]. All protocols were performed under the guidelines of the Animal Care and Use Committee, Johns Hopkins University, Baltimore, MD.

Preparation of single-cell suspension and sodium iodide treatment

Thyroid glands from the different strains of mice were collected following euthanasia. Thyroids from the same mouse strains were pooled and single-cell suspensions were prepared with a slight modification of the Lucas *et al.* method, as described previously [18]. Briefly, both lobes of thyroid glands were separated from the trachea and kept in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD, USA) at 4°C. Thyroids were digested with an enzyme solution of dispase II (1·2 U/ml) and of collagenase II (1 U/ml) [dissolved in 50 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid buffer] (Sigma, St Louis, MO, USA) and incubated in a 37°C water bath for 25 min, shaking occasionally. Cells were released from the tissue by gentle pipetting, and then washed twice with complete phosphate-buffered saline (PBS) (with 0·5 mM $MgCl_2$/0·7 mM $CaCl_2$/0·1% glucose).

Thyrocyte culture. For *in vitro* experiments, cells obtained after enzymatic digestion as described above were washed twice and cultured overnight in F12 medium with supplements (Sigma). DPI (Sigma) was used at a final concentration of 10 μM, with or without sodium iodide (NaI) for 24 h at 37°C in a 5% CO_2 chamber. For the chemiluminescence assay cells were prepared and kept in complete PBS on ice until the evaluation of ROS release. The NaI dose of 0·1 mM was determined to be optimal and used for all *in vitro* experiments (data not shown).

Reactive oxygen species detection by chemiluminescence

Luminol-derived chemiluminescence was used to assess extracellular H_2O_2 [19]. H_2O_2 release was detected in 1×10^6 thyroid cells in 2 ml of aerated complete PBS in the presence of 10 μM luminol and 10 μg/ml horseradish peroxidase (HRP) either with 0·1 mM NaI for test reactions or with saline for control reactions. Chemiluminescence was measured continuously for 60 min using a Berthold LB 9505 six-channel luminometer (Pforzheim, Germany).

Reactive oxygen species detection by flow cytometry

Mouse thyroid cells prepared as described above were resuspended in complete PBS. To detect intracellular ROS, hydroethidine (HE) was used, as it penetrates the cell membrane rapidly and enters into the cells. HE becomes fluorescent upon reduction by ROS, which can be detected by fluorescent microscopy or flow cytometry. Cells were first incubated

for 5 min with a 0·5 μM HE (Sigma) at 37°C followed by 30 min incubation with 0·1 mM NaI or PBS as control. The reaction was stopped by incubating cells for 10 min on ice followed by two washes with PBS at 300 ***g***. Phorbol 12, 13-dibutyrate (PDBu; 100 ng/ml) was used as a positive stimulator of ROS. ROS was determined within 1 h on a fluorescence-activated cell sorter (FACSCaliber) flow cytometry (BD Biosciences, Sparks, MD, USA) using fluorescent intensity of excitation at 490 nm and emission at 520 nm.

Detection of nicotinamide adenine dinucleotide phosphate oxidase/p67^{phox} by Western blot immunoassay

Single-cell suspensions of thyroids from the different mouse strains were lysed in buffer containing 50 mM Tris–HCl (pH, 7·4), 100 mM NaCl, 50 mM sodium fluoride, 5 mM ethylenediamine tetraacetic acid, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10^{-4} M phenylmethylsulphonyl fluoride, 10^{-6} M leupeptin, 10^{-6} M pepstatin A and 1% (v/v) Triton X-100 (all from Sigma). Lysates were clarified by centrifugation at 13 000 ***g*** for 10 min. Samples, each containing 20 μg of cell lysate in loading buffer, were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (12% gel) and proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA, USA). Immunoblotting was performed using monoclonal mouse anti-p67^{phox} (1:1000; BD Transduction Laboratories, San Diego, CA, USA). After washing the blots, secondary antibody bovine anti-mouse IgG HRP (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the blots. Immunoreactivity was detected by luminol-dependent chemiluminescence reagent plus (Perkin-Elmer, Rockville, MD, USA).

Statistical analysis

Data for *in vitro* work were obtained from pooled mouse thyroid glands. The experiment was performed at least three separate times. Comparisons of data were made using the non-parametric paired Student's *t*-test and were considered to be statistically significant at $P < 0{\cdot}05$.

Results

Iodine enhances ICAM-1 expression on thyroid cells of NOD.H2^{h4} mice

We have shown previously that *in vitro* treatment of NOD.H2^{h4} thyrocytes with iodine increased the surface expression of ICAM-1 on the thyrocyte cell [6]. To understand the kinetics of iodine-enhanced ICAM-1 expression, single-cell suspensions of thyroid cells from NOD.H2^{h4} mice were prepared. Thyrocytes were then incubated with 0·1 mM NaI for 24–72 h. Figure 1 shows that ICAM-1 expression increased from an average of 5% of untreated thyrocytes to >20% at 24 h. The increase was transient, as this expression decreased after 48 h and became undetectable after 72 h (data not shown). The transient nature of the phenomenon may be due to unstable mRNA, as suggested by other investigators [20]. A similar transient expression of ICAM-1 was described by Traore *et al*. when a human myeloid cell line (ML-1 cells) was challenged with a phorbol ester to initiate cell differentiation to macrophages [21].

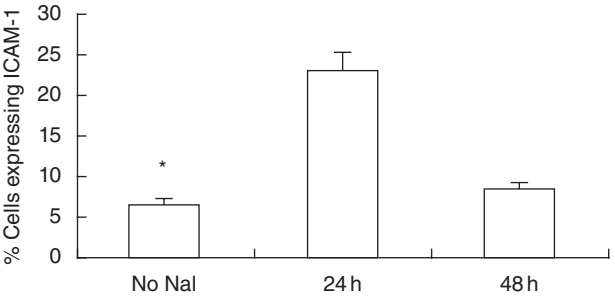

Fig. 1. Expression of intracellular adhesion molecule-1 (ICAM-1) on non-obese diabetic.H2^{h4} mouse cultured thyrocytes. Single-cell suspensions of thyroid glands were prepared and stimulated with 0·1 mM sodium iodide (NaI). Cells were assessed by flow cytometry after 24 and 48 h after iodine treatment and were compared with the no NaI group. Significantly enhanced ICAM-1 expression was noted after 24 h of iodine stimulation.

Iodine-induced ROS in NOD.H2^{h4} mouse thyroid cells

Detection of extracellular ROS by chemiluminescence. Extracellular ROS from thyroid cells was assessed by the H_2O_2-dependent luminal-derived chemiluminescence assay, which monitors H_2O_2 released from the cell. The response of NOD.H2^{h4} thyrocytes was compared with that of BALB/c. As shown in Fig. 2a, treatment of thyroid cells with iodine resulted in a marked increase in ROS levels in the NOD.H2^{h4} mice over the basal level of untreated NOD.H2^{h4} or of BALB/c thyrocytes. Increased H_2O_2 was detected within minutes of NaI addition. Maximum chemiluminescence was reached at 30 min. The level of ROS became constant in NOD.H2^{h4} mice and lasted for up to 60 min, when the determination was terminated. Thyrocytes from NOD.H2^{h4} mice exhibited a basal level of ROS expression, while this was undetectable with cells from the BALB/c mice. ROS levels in thyrocytes from BALB/c mice were undetectable throughout the experiment. Virtually no difference was seen between the untreated and iodine-treated BALB/c thyroid cells.

Detection of intracellular ROS by flow cytometry. Because ROS are generated during normal functioning of hormone synthesis in the thyroid cells, we were interested in finding:

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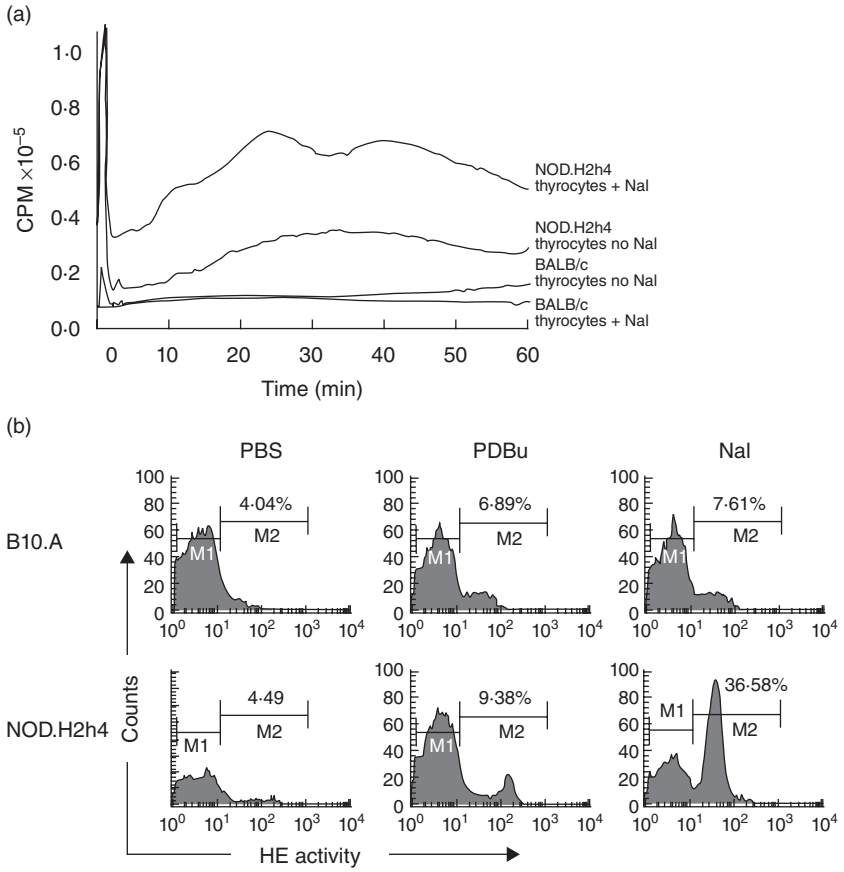

Fig. 2. (a) Luminol-derived chemiluminescence of extracellular H_2O_2 release in BALB/c and non-obese diabetic (NOD).H2^{h4} mouse thyrocytes. Single-cell suspensions from thyroid glands of 12–14 mice per group were prepared and stimulated with iodine. All samples were run in parallel, and results were compared with the no sodium iodide (NaI) control groups. Constitutively expressed levels of H_2O_2 release were noted in NOD.H2^{h4} mice that increased further after iodine addition. In contrast, levels of H_2O_2 release from BALB/c remained remarkably low at all times (representative of two separate experiments). (b) Intracellular reactive oxygen species (ROS) detection in the thyrocytes of B10.A (upper panel) and NOD.H2^{h4} mice (lower panel). Thyrocytes were stimulated *in vitro* with phosphate-buffered saline, phorbol 12, 13-dibutyrate (PDBu) or NaI. Thyrocytes of NOD.H2^{h4} but not of B10.A mice produced a spike in the level of ROS in response to iodine addition. Shown are 36·58% of thyrocytes from NOD.H2^{h4} mice and 7·61% of B10.A mice producing ROS (representative of three experiments).

(i) if this ROS burst occurred directly in response to iodine; and (ii) if thyrocytes of NOD.H2^{h4} mice produced similar levels of ROS compared with other non-susceptible strains. Single-cell suspensions of thyroid cells from NOD.H2^{h4} and B10.A mice were stimulated *in vitro* with iodine. After a brief treatment with HE as the probe (see Methods), intracellular ROS were assessed by flow cytometry.

Figure 2b shows a representative experiment of ROS detection by flow cytometry. NOD.H2^{h4} mice produced ROS rapidly within 30 min after iodine stimulation. Approximately 36·6% ROS-producing cells from NOD.H2^{h4} cells were recorded in comparison with 7·6% cells of B10.A mice. As a positive control to measure responses, PDBu was used to stimulate cellular ROS. As expected, approximately an equal number of cells responded to PDBu in both strains of mice, accounting for 6·9% of cells in B10.A and 9·4% in NOD.H2^{h4}. Thus, the number of cells producing intracellular ROS in response to iodide in NOD.H2^{h4} mice was significantly higher compared with B10.A mice.

Our results demonstrate that thyrocytes of NOD.H2^{h4} mice generated significantly increased levels of both extracellular and intracellular ROS in direct response to iodine. High levels of ROS were detected in thyrocytes from NOD.H2^{h4} mice but not in thyrocytes from BALB/c or B10.A mice.

Effect of iodine on p67^{phox} expression, a subunit of nicotinamide adenine dinucleotide phosphate oxidase

Thyroid cells are known to contain nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a well-characterized flavoenzyme [22,23]. A number of *phox* proteins participate in the active functioning of NADPH oxidase. As p67^{phox} is a critical subunit of NADPH oxidase, we investigated the effect of iodine on the expression of p67^{phox} protein in thyroid cells. Thyroid cells from 8- to 10-week-old NOD.H2^{h4} or BALB/c mice were isolated and treated for 0 min (control), 15 min and 30 min with 0·1 mM iodine, as was used in the ROS release detection experiments. The cells were then digested and expression of p67^{phox} protein was detected by Western blot assay. As demonstrated in Fig. 3, NOD.H2^{h4} mice expressed p67^{phox} protein in their thyroid cells prior to iodine treatment. In contrast, BALB/c thyroid cells expressed no p67^{phox} before treatment with iodine, which remained undetectable even after 15 min. However, p67^{phox} was detectable after 30 min of iodine treatment. The 30-min iodine-induced p67^{phox} expression in BALB/c was fairly low and was not comparable with the constitutive expression of p67^{phox} of thyrocytes from the NOD.H2^{h4} mice.

Cytokines such as IFN-γ are known to affect the outcome of autoimmune thyroid disease [6,24,25]. Therefore we

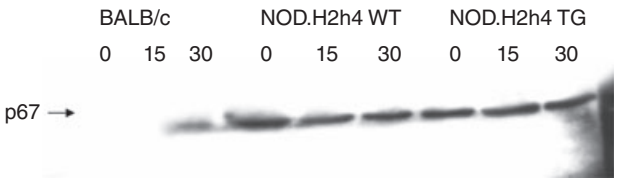

Fig. 3. Expression of cellular protein p67^{phox}, a component of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that triggers reactive oxygen species release in cells. Non-obese diabetic (NOD).H2^{h4} mouse thyroid cells were compared with those of BALB/c. As shown, NOD.H2^{h4} mice constitutively expressed p67^{phox} prior to iodine treatment. In contrast, BALB/c mice did not constitutively express p67^{phox}.

analysed the p67^{phox} expression in the NOD.H2^{h4} transgenic mice that expresses IFN-γ in their thyroid cells. The IFN-γ transgenic mice, like the parental NOD.H2^{h4}mice, expressed p67^{phox} constitutively. Iodine treatment for 15–30 min did not show any remarkable changes in the expression of p67^{phox} (Fig. 3). Thus, elevated expression of p67^{phox}, a subunit of NADPH oxidase, is associated with the constitutive ICAM-1 expression.

Diphenyleneiodium inhibits iodine-induced ROS and ICAM-1 expression in thyroid cells

Our results demonstrate that iodine treatment enhanced ROS production in thyrocytes from NOD.H2^{h4} mice but not in any of the non-susceptible strains. However, to confirm that ROS were generated by the enhanced activity of NADPH oxidase in NOD.H2^{h4} mice, DPI was used to inhibit enzymes that generate ROS [26].

To address this issue, a single-cell suspension of NOD.H2^{h4} mouse thyroid cells was incubated with 10 μM DPI for 24 h before the addition of NaI and compared with untreated cells. The data in Fig. 4a show a representative experiment illustrating a reduction in the ROS-mediated fluorescence intensity of HE (330 *versus* 220) in the DPI-treated thyrocytes compared with DPI-untreated cells.

Next, we asked whether ROS inhibition reduced ICAM-1 protein expression on the cell surface. Therefore, we assessed ICAM-1 on thyrocytes treated with DPI as in the ROS experiments. The data presented in Fig. 4b show an almost threefold decrease in the mean fluorescence intensity of ICAM-1 expression (199 *versus* 74) after overnight treatment with DPI in the presence of NaI. The results illustrated in Fig. 4a and b support our hypothesis that iodine may increase ICAM-1 through the influence of ROS on the cellular signalling system leading to ICAM-1 expression.

Discussion

We have reported previously that the NOD.H2^{h4} mouse develops AT spontaneously and expresses ICAM-1 constitutively on the thyrocytes. ICAM-1 expression is enhanced further by supplementing the diet with iodine [6]. The experiments performed in this report attempt to understand and explain the mechanisms responsible for this observation. As increased thyroidal ICAM-1 could be a risk factor in the initiation and progression of AT in genetically susceptible individuals, as well as in certain mouse strains, understanding the mechanism by which it occurs could lead to new therapies in patients with Hashimoto's thyroiditis. Elevated levels of ICAM-1 or ICAM-1 mRNA in humans have been shown by several investigators studying thyrocytes from Hashimoto's thyroiditis patients [15,27,28]. While these data suggest that ICAM-1 plays an important role in autoimmune thyroid disease, it is difficult to determine in humans if it is a cause or consequence of disease in patients. Previous work by other investigators using another model of iodine-enhanced AT, the obese strain (OS) chicken, has implicated ROS as a participant in disease pathogenesis

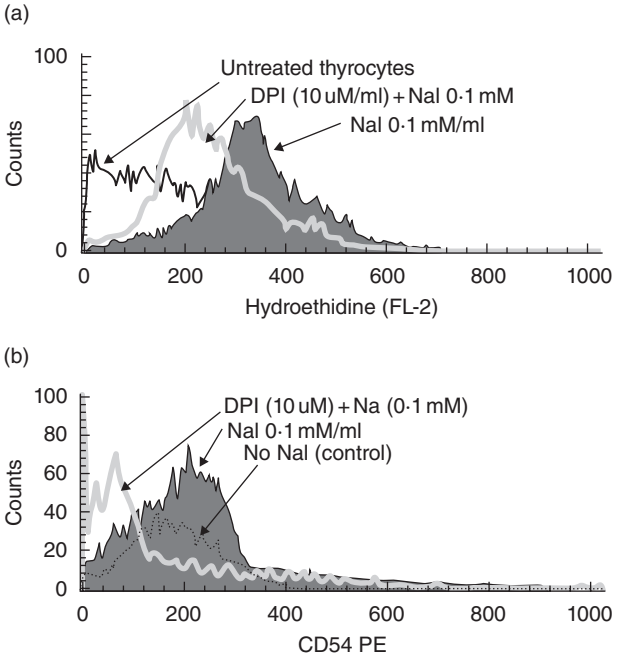

Fig. 4. (a) Effect of diphenyleneiodium (DPI) treatment on the reactive oxygen species (ROS) generation by thyrocytes of non-obese diabetic (NOD).H2^{h4} mice. A remarkable decrease in the intracellular ROS production was noted after treatment with DPI, a pharmacological inhibitor of enzymes that generate ROS. The solid histogram represents the intracellular ROS in response to sodium iodide (NaI) alone, and the open histogram shows the decreased fluorescence intensity (330 *versus* 220 respectively) after DPI treatment. The broken line histogram represents control (unstimulated and untreated) cells. (b) Effect of DPI treatment on adhesion molecule-1 (ICAM-1) expression on NOD.H2^{h4} mouse thyrocytes. ICAM-1 expression decreased after treatment with DPI. The solid histogram represents ICAM-1 in response to NaI alone, while the open histogram shows the decreased fluorescence intensity (199 *versus* 77 respectively) after DPI treatment. The broken line histogram represents control (unstimulated and untreated) cells.

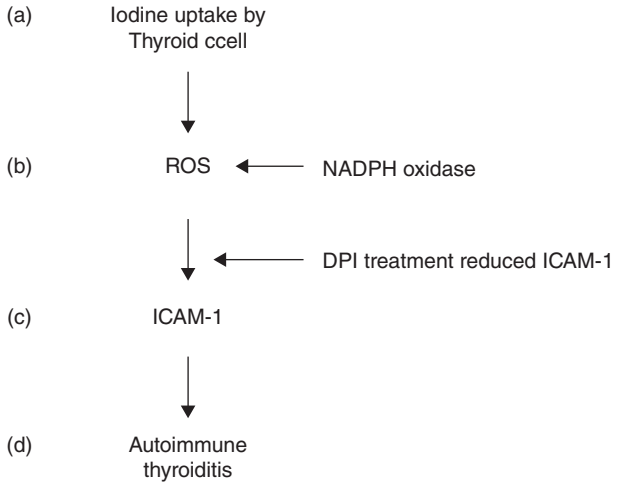

Fig. 5. Proposed cascade of iodine-enhanced cellular events leading to adhesion molecule-1 (ICAM-1) up-regulation with subsequent autoimmune thyroiditis. (a) Active uptake of excess iodine. (b) Generation of reactive oxygen species (ROS) induced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [inhibited by diphenyleneiodium (DPI)]. (c) ICAM-1 up-regulation because of the ROS spike (inhibited by DPI). (d) Mononuclear cell infiltration and disease.

[29,30]. While no information about ICAM-1 expression in the OS chicken thyroid is available, the investigators delayed thyroiditis by using anti-oxidants, thus linking the generation of ROS to the pathogenesis of autoimmune thyroid disease [30].

Based on the results of the current study and in conjunction with our previous work, we propose a series of steps following the excess iodine uptake by thyrocytes of the NOD.H2^{h4} mouse leading to ROS generation and up-regulated expression of ICAM-1. The proposed events of ICAM-1 expression following iodine exposure are summarized in Fig. 5. Iodine is taken up by the thyroid epithelial cell (Fig. 5a). A spike in the ROS generation occurs because of the activity of the already-present enzyme NADPH oxidase (Fig. 5b). While the ROS spike, which includes H_2O_2 production, promotes organification of the iodine on the thyroglobulin molecule, it may also trigger signal transduction resulting in increased ICAM-1 expression on the surface of the thyrocyte (Fig. 5c) [31]. Thus, an environment is created within the thyroid gland combining a strongly immunogenic antigen, highly iodinated Tg, with an increased adhesion molecule, ICAM-I, favouring the retention of circulating lymphocytes, their activation and subsequent cytokine release which, over time, develops into AT (Fig. 5d) [2,6,32].

Evidence from this study and from previous work supports this model. Within 15–30 min after *in vitro* exposure of the thyrocyte to iodine there is an increase of ROS activity (shown in Fig. 2) because of a constitutive expression of NADPH oxidase (Fig. 3). These changes were evident only in thyrocytes of the NOD.H2^{h4} mouse (Fig. 5b and c). This, by itself, is an unusual event, as thyroid epithelial cells are normally protected from acute exposure to large amounts of iodine by the Wolff–Chaikoff effect.

This effect relates to an inhibition of iodide uptake and organification after exposure to high iodine levels [33]. We observed this effect in the BALB/c but not in the NOD.H2^{h4} mouse (Fig. 2a). Thus, based on our observation, we suggest that the Wolff–Chaikoff effect appears to be entirely ineffective or has a different threshold in the NOD.H2^{h4} mouse.

Nicotinamide adenine dinucleotide phosphate oxidase expression has been recognized in thyroid cells for some time [22] but its role, although indirect, in thyrocyte ICAM-1 up-regulation has not been described previously. NADPH oxidase functioning is regulated by the assembly of several members of the *phox* protein family. The initiating subunit p47^{phox} is located in the resting cells and upon stimulation mediates translocation of p67^{phox} to the cell membrane in association with the cell cytoskeleton [34,35]. These levels of the p67^{phox} subunit of NADPH oxidase serve as a surrogate for the entire enzyme complex and may relate directly to the increased ROS generation following excess iodine exposure. The increased ROS leads to increased ICAM-1 expression. It was interesting to note that, like constitutive ICAM-1 expression, the p67^{phox} expression was also found to be expressed constitutively prior to iodine stimulation, but only in the susceptible NOD.H2^{h4} mice and not in the non-susceptible BALB/c mice. Several species of ROS are generated during the metabolic functioning of normal thyroid cells; however, it is not clear if only one species is enough to induce the up-regulation of ICAM-1 or more than one species are required. While we have no evidence that NADPH oxidase is defective, the constitutive expression of this enzyme within the thyrocyte prior to iodine stimulation *in vitro* suggests an abnormality somewhere along this pathway.

Another point to consider when comparing the susceptible NOD.H2^{h4} mouse with the non-susceptible BALB/c mouse is a potential toxic effect of the higher level of H_2O_2 on the NOD.H2^{h4} thyrocyte. A direct response to iodine was demonstrated by the luminol-derived chemiluminescence experiment (see Fig. 4a), where thyroid cells were assayed for generation of extracellular H_2O_2. This may damage the cell further and lead to release of degraded thyroglobulin, as described by Duthoit *et al.* [36], potentiating the autoimmune response further because of its increased iodinated epitopes [32].

Our data demonstrate clearly that iodine by itself can induce ICAM-1 in NOD.H2^{h4} mouse thyrocytes following ROS generation. As is evident from these data, none of the non-susceptible mouse strains following iodine exposure expressed such elevated levels of ROS. It is not a question of non-responsiveness of the thyrocytes altogether, as we showed that thyrocytes from BALB/c mice generate ROS in response to PDBu as an alternate stimulator. Thus, while thyrocytes from BALB/c mice produce ROS equally to the NOD.H2^{h4} after PDBu stimulation, increased ROS because

of excess iodine exposure is not seen (Fig. 2b). It was shown only in the NOD.H2^{h4} mouse, in which both ICAM-1 and NADPH oxidase were present constitutively. The poor ROS generation in the non-susceptible BALB/c mice correlates well with the low ICAM-1 expression, as we have shown previously that dietary supplementation of iodine for several weeks did not enhance ICAM-1 expression in BALB/c mouse thyrocytes {3596}.

The association between ROS generation and ICAM-1-enhanced expression was supported further by the experiments using DPI. DPI inhibits ROS generation mediated by NADPH oxidase {3439}. Our results using DPI showed a remarkable decrease in the fluorescent intensity of ROS and the subsequent expression of ICAM-1.

In the present study we also show that DPI, an inhibitor of enzymes that generate ROS, decreased the surface ICAM-1 protein markedly on the thyroid cells of NOD.H2^{h4} mice. Pretreatment with other inhibitors specific for other mediatory proteins may also be useful in understanding the signalling cascades of ROS-mediated up-regulation. The ROS-dependent up-regulation of ICAM-1 pathway provides one potential mechanism for promoting the autoimmune process leading to AT. While the up-regulated ICAM-1 may not be the sole mechanism that regulates disease progression, it is definitely an important disease initiating factor. The use of blocking antibodies in humans may not be practical so utilizing another pathway, such as blocking thyroidal ROS, to achieve the same end may be a suitable alternative approach. ICAM-1 inhibition with anti-oxidants, or other compounds that inhibit the enzymes generating ROS, could lead to new therapies that delay or reverse disease.

Acknowledgements

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