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RESEARCH ARTICLE

Leukocyte Nurr1 as peripheral biomarker of early-life environmental exposure to permethrin insecticide

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Abstract
The effect of a low dose of the insecticide permethrin administered during early-life was evaluated on leukocytes inflammation mediators on 300- and 500-day-old rats. Nurr1, NF-κB-p65, Nrf2, lipid peroxidation and GSH levels increased with age but compared to the control group, treatment with permethrin induced a significant increase only of Nurr1 and lipid peroxidation in oldest rats. TNF-α and Rantes increased, while IL-1β, IL-2, IL-13 decreased in oldest treated rats. The results propose Nurr1, TNF-α, Rantes, GSH and plasma lipid peroxidation as peripheral biomarkers for monitoring the impact of early-life environmental exposure to xenobiotics in old age.

Keywords: Nurr1, NF-κB, Nrf2, leukocyte, cytokines, permethrin, rat

Introduction

Early life is a developing phase, which is particularly vulnerable to the effects of adverse environmental exposures because many different pathways, as well as structure and function of several macromolecules, are programmed during this period and for this reason, early life can influence the program of gene expression in adulthood (Oliveira et al. 2011; Vaiserman, 2011). Accordingly, rats exposed during early life to endocrine disruptors have lifelong effects on neuroendocrine gene expression, together with progression of senescence (Gore et al. 2011). Furthermore, health status in adulthood also depends on early postnatal nutrition (Gabory et al. 2011): in fact diet, drugs and the environment mediate changes that maintain the memory of exposure and produce long lasting effects, when exposure has ended.

Our previous studies underline the correlation between early life environmental exposure and the development of long-time effects (Nasuti et al. 2007; Vadhana et al. 2011; Carloni et al. 2012). In fact, when rats were exposed during early life to permethrin pesticide, they showed central and peripheral impairment of redox and immune systems. Increased dopamine turnover and protein oxidation together with decreased GSH level in striatum of adolescent rats were observed, while lower erythrocyte glutathione peroxidase activity and reduction in superoxide anion production during monocyte respiratory burst were measured (Nasuti et al. 2007). Recently, we demonstrated that early life exposure to permethrin induces neurodegeneration and inflammation in adult rats with an important increase in plasma of pro-inflammatory cytokines (IL-1β, IL-2, IFN-γ and rat-Rantes) (Carloni et al. 2012; Vadhana et al. 2011).

Since upon cited markers, related with the redox and inflammation systems, were increased, here leukocytes together with other plasma parameters were chosen to investigate their potential role as biomarkers of early life exposure to environmental pesticides. Leukocytes are a good choice as cell markers because first of all they are extremely sensitive to the environment and contaminants accumulated through the food chain, and these influence gene expression during immune differentiation. Secondly, they could represent indicative indices of alterations that have occurred in other tissues,

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and finally they can be obtained without any invasive procedure for animals (Nasuti et al. 2007; Prescott & Saffery 2011). Three transcription factors, Nurr1, NF-κB and Nrf2, were chosen because of their involvement in the regulation of genes modulating the proinflammatory response, redox and immune systems together with some corresponding indicators (i.e. GSH, SOD, lipid peroxidation) that can monitor the redox system status.

Nurr1 is a transcription factor belonging to the NR4A family that plays an essential role in physiological processes (i.e. development of dopaminergic neurons, apoptosis in lymphocytes and other cell types) and it has a key role in inflammation (Lin et al. 2004). Nurr1 is highly inducible in macrophages by inflammatory cytokines and oxidized lipids such as oxidized LDL (Pei et al. 2006). Moreover, its gene expression is increased by enhanced NF-κB and CREB-1-binding activity on the gene promoter and also by stimulation of pro-inflammatory cytokines (i.e. IL-1β and TNFα) or PGE_2 (Murphy et al. 2001; McEvoy et al. 2002).

In order to evaluate the impact of permethrin exposure during early life on adulthood, we examined the effects of the insecticide administration from 6th to 21st day of life on NF-κB, Nrf2 and Nurr1 leukocyte transcription factors and on system mediators related to inflammatory and redox status (e.g. plasma cytokines, GSH, SOD,) in 300- and 500-day-old rats, that correspond to 25- and 50-year-old humans (http://www.ratbehavior. org/RatYears.htm), representing respectively, adult age and the beginning of oldness.

Materials and methods

Chemicals
All reagents were of pure and analytical grade and were obtained from Sigma Chemical Co. (USA). Technical grade (75:25, trans:cis; 94% purity) 3-phenoxybenzyl-(1R,S)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylate, Permethrin was generously donated by Dr. A. Stefanini of ACTIVA, Milan, Italy.

Animals
Male and female Wistar rats from Charles River (Calco, LC, Italy), weighing 250–270 g and about 90 days old were used. The animals were housed in plastic (Makrolon) cages (five rats/cage) in a temperature controlled room ($21 \pm 5°C$) and maintained on a laboratory diet with water ad libitum. The light/dark cycle was from 7 p.m. to 7 a.m. Animal use in this study complied with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Rat pups born in our laboratory from primiparous dams were used in the study. The parturition day was set as Post Natal Day 0 (PND0). On PND1, all litters were examined externally for the presence of gross abnormalities, sexed, weighed and the female pups were discarded. Two male pups were assigned to each dam until weaning (PND21). No

cross-fostering was employed. At 2 days of age, litters were randomly assigned to two experimental groups ($n = 7$ rats for each).

Treatment
Permethrin was dissolved in corn oil and administered orally by an intragastric tube (4 mL/kg) at a dose of 1/50 of LD_{50} corresponding to 34.05 mg/kg (Agency for Toxic Substance and Disease Registry, 2005). The dosage was chosen by considering the "no observed adverse effect level" (NOAEL) for permethrin of 25 mg/kg. The compounds were administered once a day in the morning from PND6 to PND21. Control rats were treated with vehicle (corn oil 4 mL/kg) on a similar schedule. The volume of the compound administered was adjusted daily based on body weight measured during the dosing period. On PND21, the offspring were weaned and the littermates were housed together. At adult age (PND 300) and old age (PND 500), seven rats from each group (permethrin treated and control groups) were sacrificed by exposure to CO_2, and their blood was collected.

Leukocytes extraction and preparation
Leukocytes from seven control and seven treated rats of both adult and old groups, were isolated using Mono-Poly Resolving Medium (ICN, Biomedicals, Milan, Italy) and pooled. They were then recovered from gradients and washed using PBS and lysated using RIPA buffer [1% NP40, 0.5% Na-deoxycolic acid and 0.1% SDS in phosphate buffered saline (PBS)] with fresh added protease inhibitors. The lysates were passed several times through a 22-gauge needle in order to shatter the DNA molecules.

Western blot
Leukocyte lysates obtained as described above were quantified using the Lowry method (Lowry et al. 1951) and equal amounts (30 μg) of lysate were separated using SDS–PAGE (10%) and electrophoretically blotted on a nitrocellulose support (Hybond C, Amersham Bioscience, Little Chalfont, UK).

For each experiment, reactive sites were then blocked with PBS containing 0.05% Tween 20 and bovine serum albumin (10%) for 1 h at room temperature and incubated respectively with polyclonal Nurr1 (diluted 1:100), polyclonal Nrf2 (diluted 1:200) and polyclonal NF-κB p65 (diluted 1:200) anti-rabbit antibodies (Santacruz Inc, USA) and then with secondary anti-rabbit antibody diluted 1:5000 (KPL, USA). β-actin was utilized as a control for equal protein loading: membranes were stripped and reprobed for β-actin with an anti-rabbit monoclonal antibody (Abcam plc, UK) diluted 1:3000. Antibodies were diluted in PBS containing 0.05% Tween 20 and bovine serum albumin (2%). Every gel was loaded with molecular weight markers including proteins with MW from 250 to 4 kDa (Invitrogen, USA). HeLa cells lysate was used as positive control (data not shown). Image capturing was performed using the KODAK Image Station 2000r Systems.

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Cytokines levels in plasma

IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IFNγ, TNFα, GM-CSF and Rat Rantes levels were determined in triplicate in pooled plasma, using commercially available ELISArray Kits Multi-Analyte for Rats (QUIAGEN Company, USA) according to the manufacturer's protocol. The plate included a standard curve and known positive and negative controls. Absorbance was read at 450 nm and at 570 nm using a microtiter ELISA reader. The limit of detection for cytokines is 0.01 ng/mL.

GSH levels in plasma

Glutathione (GSH) content in plasma (100 μL) obtained from control and treated rats was assessed by the method previously described by Butler et al. (Butler et al. 1994). Oxidized GSH was measured using the sulphydryl reagent 5,5′-dithio-bis(2-nitrobenzoic acid) on a spectrophotometer at the wavelength of 412 nm with respect to its standard.

SOD levels in plasma

Superoxide dismutase (SOD) was determined according to the method of Misra and Fridovich (Misra & Fridovich, 1972). SOD level was monitored in a reaction mixture containing 0.05 M carbonate buffer, 0.1 M EDTA, pH 10.2, 0.05 M adrenaline and 50 μL of plasma from control and treated rats. The change in absorbance was recorded spectrophotometrically at 480 nm at 37°C. A standard curve was used to report the results as μg SOD/mL plasma.

Lipid hydroperoxide detection

Intracellular lipid hydroperoxides were detected using a membrane-localized fluorescent probe DPPP that reacts specifically with hydroperoxides and becomes highly fluorescent when oxidized (Takahashi et al. 2001). Plasma from control and treated groups (50 μL) was incubated with 1 μM DPPP in 1 mL PBS at 37°C for 5 min in the dark. The fluorescence intensities of the samples were measured with a Hitachi 4500 spectrofluorimeter using 351 and 380 nm as excitation and emission wavelengths, respectively. Results are expressed as fluorescence intensity (arbitrary units).

Statistical analysis

Data are expressed as mean values ± SEM ($n = 7$). Each experiment was performed in triplicate and repeated three times. Statistical analysis was carried out using a two-way ANOVA with one factor within (time: adult and old age) and one factor between (treatment: control and PERM) for GSH, SOD and lipid peroxidation plasma analysis and one-way ANOVA was used for cytokines, all followed by post-hoc analysis (Statsoft Statistica Software, 9.0). A p value <0.05 was considered statistically significant.

Results

Western blot

Figure 1 shows the western blot analysis of pooled leukocyte homogenates obtained from control and treated rats aged 300 and 500 days with anti-Nurr1, anti-Nrf2 and anti-NF-κB p65 antibodies. Western blot of NF-κB p65 shows two bands with the same intensity as reported by Konturek (Konturek et al. 2008). No differences were observed between 300 day old control and treated groups in Nurr1, NF-κB p65 and Nrf2 protein levels, while an increased Nurr1 protein level was observed in the treated group at 500 days with respect to its control. To confirm the Nurr1 results obtained using 30 μg of protein, experiments were repeated using different protein concentrations: samples from adult rats showed no changes between control and treated rats at every tested protein concentration (data not shown), while results from old rat samples, tested also at 50 μg of protein, confirm the increased expression of Nurr1 in treated samples (Figure 1). No changes were observed for the other protein levels. As can be seen, the NF-κB p65 and Nrf2 protein levels of both control and treated groups at 500 days are higher than their respective counterparts at 300 days. For this reason, in order to discriminate the difference between control and treated rats at 500 days, the NF-κB p65 analysis was repeated using lower protein concentrations (7.5 and 15 μg).

Cytokine levels in plasma

Figure 2 shows the cytokines levels in plasma of control and treated groups at 500 days. A significant decrease in IL-1β plasma level (0.10 ± 0.00 ng/mL plasma) was measured in treated rats with respect to the control (0.29 ± 0.02 ng/mL plasma) ($p < 0.05$). The same behavior was observed for IL-2 (1.02 ± 0.13 for control and 0.43 ± 0.17 ng/mL plasma for treated, $p < 0.05$), and IL-13 (0.70 ± 0.05 for control and 0.30 ± 0.00 ng/mL plasma for treated, $p < 0.05$), while on the contrary, there was an increase in Rat Rantes in plasma of treated rats (2.69 ± 0.18 ng/mL plasma) with respect to the control ones (2.12 ± 0.04 ng/mL plasma) ($p < 0.05$). TNF-α was detected only in plasma of treated rats (0.06 ± 0.04 ng/mL), ($p < 0.05$) while IL-1α, IL-4, IL-6, IL-10, IL-12, IFNγ and GM-CSF concentrations in both control and treated groups were not quantified because under the limit of kit detection (0.01 ng/mL).
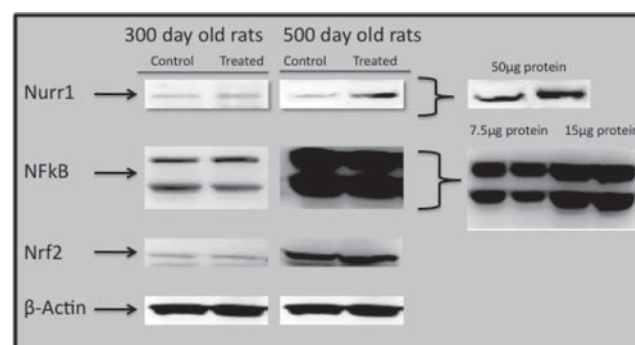

Figure 1. Western blotting detection of Nurr1, NF-κB p-65, Nrf2 protein expression on leukocytes of control and treated rats in adult and old age. The immunoblot shown in the figure is representative of three replicated experiments using pools from seven control and seven treated rats from each age group.

GSH, SOD and lipid peroxidation levels in plasma

In Table 1, GSH, SOD and lipid peroxidation plasma levels of 300- and 500-day-old rats are reported. A significant decrease in GSH level was measured with respect to the control (76.23 ± 0.65 for control and 48.31 ± 1.30 μg/mL plasma for treated, $p < 0.05$) at 300 days, while no differences were observed between control and treated at 500 days, despite their levels being significantly higher (167.79 ± 3.90 for control and 182.72 ± 1.95 μg/mL plasma for treated, $p < 0.05$) than those in the adult groups. No significant differences were observed on SOD levels for control and treated 500-day-old rats.

A significant decrease in lipid peroxidation was obtained in treated rats with respect to the controls (1248 ± 5.29 for control and 1065 ± 1.12 fluorescence intensity for treated, $p < 0.05$) at 300 days. The opposite trend was observed for 500-day-old rats (3131 ± 5.20 for control and 5611 ± 15.86 fluorescence arbitrary units for treated, $p < 0.05$) and their levels were significantly higher than those found at 300 days.

Discussion

Permethrin, which belongs to the insecticide family of pyrethroids, was the first synthetic pyrethroid characterized by high photostability and is the most used pyrethroid in USA and in many other countries (Agency for Toxic Substance and Disease Registry, 2005). Permethrin's human impact derives from its use as a fungicide, for wood preservation and children may absorb this insecticide by touching treated surfaces or from lice shampoos or scabies treatments (Barr et al. 2010). Moreover, urine permethrin metabolites in humans confirm the use of this insecticide, therefore its impact on human health should be object of investigation (Barr et al. 2010). Indeed, pesticides such as pyrethroids have a relevant role in the depression of the immune system because they can greatly induce leukocytosis, decrease NK-cell counts, T- and NK-cell functions and they can increase the CD4+/CD8+ ratio (Segerstrom & Miller 2004).

In the present study, permethrin was administered only during the neonatal period and no trace of the pesticide was present in the animals during adulthood and oldness, therefore excluding any direct mechanism of action (Nasuti et al. submitted).

In our animal model, in the 500-day-old rats, both control and early life permethrin treated groups showed increased NF-κB p65 protein expression with respect to the groups in adult age. As reported by some authors
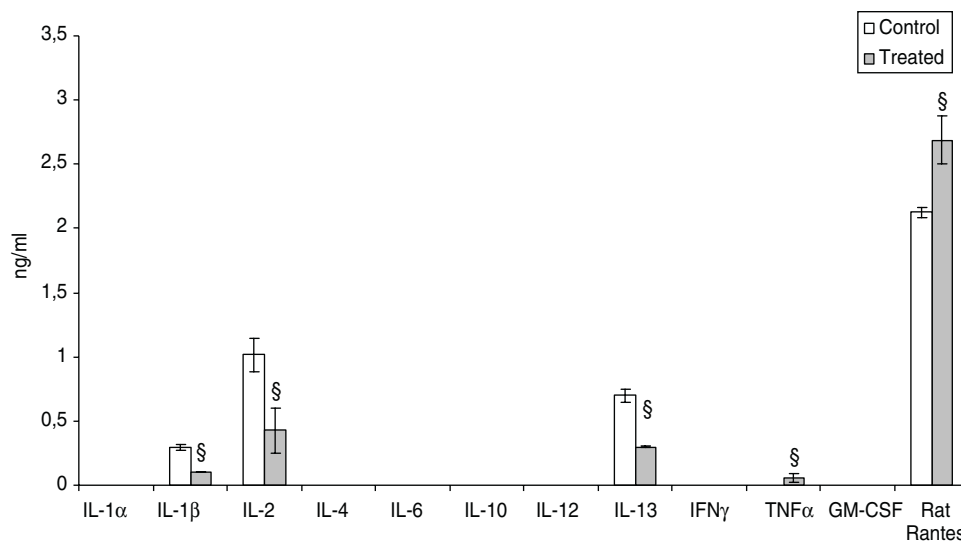

Figure 2. Cytokine levels (ng/mL) in pooled plasma obtained from seven control and seven treated rats sacrificed in old age. §$p ≤ 0.05$ vs control. Data are expressed as mean values ± SEM of experiments performed in triplicate.

Table 1. GSH, SOD and lipid peroxidation levels in pooled plasma from seven control and seven treated rats at 300 and 500 days.

	300-day-old rats		500-day-old rats	
	Control	Treated	Control	Treated
GSH (μg/mL plasma)	76.23 ± 0.65	48.31[b] ± 1.30	167.79[a] ± 3.90	182.72[a] ± 1.95
SOD (μg/mL plasma)	*	*	10.65 ± 0.75	11.00 ± 0.40
Lipid peroxidation (arbitrary units)	1248 ± 5.29	1065[§] ± 1.12	3131[a] ± 5.20	5611[a,b] ± 15.86

Data are expressed as mean values ± SEM of experiments performed in triplicate.
[a]$p ≤ 0.05$ vs 300-day-old rat.
[b]$p ≤ 0.05$ vs respective control.
*Previously published by Carloni et al. (2012).

(Salminen & Kaarniranta 2010), in old age, chronic activation of NF-κB has the capacity to induce the senescent phenotype by increasing inflammatory and homeostatic responses such as apoptosis, autophagy, and tissue atrophy and this may explain why NF-κB protein levels were higher in old age compared to adult age rats. However, differences in NF-κB protein expression between control and treated groups were not observed in both 300- and 500-day-old rats, demonstrating that the increased NF-κB was only due to the aging process. This outcome is in agreement with the age related increase in NF-κB previously reported in different tissues (Helenius et al. 2001; Salminen et al. 2008; Lee et al. 2010). On the contrary, changes between treated and control rats were present in the cytokine plasma profile of the oldest group. In particular, a major decline in the immune system in treated rats versus control was noted by analyzing IL-1β, IL-2 and IL-13 that were significantly decreased in the treated group.

TNF-α and rat Rantes, two important proteins that are instrumental in recruiting inflammatory cells such as T cells and monocytes/macrophages, were increased in treated versus control groups. It has been demonstrated (Wen et al. 2010) that TNF-α mediates the induction of protein expression for rat Rantes that plays a central role in the development of atherosclerosis through its chemoattractant function on monocyte cell recruitment for atherosclerotic plaques. Previous studies on *Resus macaques* showed that early life smoke exposure increased gene and protein expression of Rantes in adulthood, supporting its role as a predictor inflammatory biomarker (Villablanca et al. 2010). Furthermore, it has been demonstrated in rats that Rantes can play a role in mediating the progression from acute to chronic inflammation (Ajuebor et al. 2001). Overall, the decline in the immune system in the oldest rats is evident when their cytokine plasma profile is compared with that of 300-day-old rats (Vadhana et al. 2011). In fact, in 500-day-old rats, IL-1α, IL-4, IL-10, IL-12, IFNγ and GM-CSF were under the detectable levels (0.01 ng/mL), whereas in 300-day-old rats, IL-1β, IL2, rat-Rantes, IFNγ, and IL-10 increased in all treated samples with respect to the control ones and IL-10 and IFNγ were found only in the treated samples (Vadhana et al. 2011). Instead, the higher chemokine Rantes and TNF-α levels in 500-day-old treated rats suggest a worst chronic inflammatory state with respect to control rats.

Here, no differences between control and treated groups on leukocyte Nurr1 protein expression were observed during adulthood, while an increase was detected in the oldest group. This outcome cannot be ascribable to the NF-κB induction, but it can be correlated with the increased TNF-α levels according to Takayasu et al. (2010). Although the mechanisms involved in long-term changes in gene expression need to be investigated in future studies, the increased Nurr1 level in leukocytes together with changes in pro-inflammatory cytokines, suggests that they may be considered as good biomarkers

of premature senescence reflecting the alterations that occur during the aging process in our animal model. As reported in previous studies, in age-related diseases such as rheumatoid arthritis and osteoarthritis, Nurr1 is highly expressed in macrophages, suggesting that this transcription factor plays a pivotal role in chronic inflammatory diseases (McEvoy et al. 2002; Pei et al. 2006). At this point, we can hypothesize that the higher production of pro-inflammatory cytokines (IL-1β, IL-2, IFN-γ, rat-Rantes) observed in adulthood in the same cohort of treated rats (Vadhana et al. 2011) could be implicated in the progressive enhancement, in older age, of Nurr1 expression as an adaptive response to the inflammatory process.

Another biomarker of aging is represented by the increased plasma lipid peroxidation found in 500-day-old treated rats due to overproduction of ROS, correlated with permethrin's capability to induce premature senescence as previously reported (Nasuti et al. 2003, 2007; 2008; Gabbianelli et al. 2004; 2009a; 2009b). In response to increased oxidative stress, the antioxidant system of treated oldest rats failed to activate, maybe for its weakness due to oldness, as demonstrated by unchanged GSH and SOD levels in plasma. Consistent with this last finding, Nrf2 signaling essential for the activation of a wide range of antioxidant enzymes (Jeong et al. 2006; Surh et al. 2008), was no longer activated by increased plasma lipid peroxidation in the treated oldest group compared to the control one, although GSH levels were significantly increased with respect to those measured in both adult groups. This last data is unusual because it is generally reported that aging is correlated with a decrease of GSH levels; however, it could be hypothesized that old rats increase their GSH content as a failed attempt to contrast the overproduction of ROS due to aging, confirmed by the increase in lipid peroxidation and Nf-κB protein expression (Yazihana et al. 2008).

In conclusion, early life permethrin treatment (from 6 to 21 days of life) has permitted to develop an animal model displaying age-related changes that mirror those seen in humans concerning the immune and ROS detoxification systems, and that could be usefully employed to set-up preventive therapeutic approaches.

Amongst all the investigated biomarkers, increased leukocyte Nurr1, TNF-α, Rantes, lipid peroxidation and GSH plasma content could represent key biomarkers useful for monitoring the impact of early life environmental factors on the immune system in old age, and indirectly, they could give information on the state of other organs (i.e. brain, heart) that cannot be isolated and analyzed.

Studies to investigate the mechanisms related with the observed changes will be the object of future investigations.

Declaration of interest

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