Full Paper

Effect of Nicotine-Induced Corticosterone Elevation on Nitric Oxide Production in the Passive Skin Arthus Reaction in Rats

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Abstract. To elucidate the anti-inflammatory action of nicotine-induced corticosterone elevation on the passive skin Arthus reaction (PSAR), we investigated the inflammatory process in the PSAR. The polymorphonuclear leukocyte (PMNs) infiltration was observed just before as well as after elicitation by measuring extractable myeloperoxidase. The plasma exudation was significantly inhibited by anti-rat tumor necrosis factor (TNF)- α antibody (5 μ g/site, i.d.) at the time of sensitization or by superoxide dismutase (52500 units/kg, i.p.) 1 h before elicitation or *N*^G-nitro-L-arginine-methyl ester (100 mg/kg, i.v.) just at elicitation. Pretreatment with a single injection of nicotine (0.8 mg/kg, i.p.) 30 min before elicitation suppressed the plasma exudation but not the PMNs infiltration. This nicotine-induced decreasing effect was abolished in animals supplemented with L-arginine (300 mg/kg, i.v.) just at elicitation. The production of nitric oxide (NO) in peritoneal PMNs derived from an animal injected peritoneally with oyster glycogen was significantly suppressed by pretreatment with nicotine (0.8 mg/kg, i.v.) 30 min prior to harvesting. This inhibitory action of nicotine was abolished in animals pretreated with mifepristone (30 mg/kg, s.c.), a glucocorticoid receptor antagonist. These findings indicate that a single systematic administration of nicotine may attenuate the plasma exudation in the PSAR by suppressing the production of NO in the PMNs primed with TNF- α via nicotine-induced endogenous glucocorticoid.

Keywords: passive skin Arthus reaction, nicotine, tumor necrosis factor- α , superoxide, nitric oxide

Introduction

It is known that the hypothalamic-pituitary-adrenal (HPA) axis has an important role in the regulation of inflammation through the anti-inflammatory action of glucocorticoids produced in the adrenal cortex (1–4). Some reports have shown that the activation of the HPA axis is driven by stimulation of the cytokines, including interleukin (IL)-1, tumor necrosis factor (TNF)- α , and IL-6, on the paraventricular nucleus (PVN) (5–7). It has also been shown that nicotine is a potent activator of the HPA axis, stimulating the release of serum corticosterone and adrenocorticotropin (8, 9). The proposed mechanism of activation of the HPA axis by nicotine is that nicotine indirectly stimulates the release of

corticotropin-releasing hormone from the parvocellular region of the PVN of the hypothalamus (10) and/or from corticotropin-releasing factor-containing neurons in the median eminence (11). In previous studies, nicotine has been shown to inhibit the plasma extravasation induced by the inflammatory mediator bradykinin (12). In addition, the effect of nicotine on bradykinin- and platelet activating factor-induced plasma extravasation was attenuated by adrenal medullectomy, pretreatment with naloxone, β 2-blockers, glucocorticoid receptor antagonists, and glucocorticoid synthesis inhibitors (13). Recently, we have demonstrated that a single systematic administration of nicotine inhibits the plasma exudation in the immunological inflammation model, the passive skin Arthus reaction (PSAR), via serum corticosterone elevation. This action is induced by activation of the PVN through the brain nicotinic acetyl-

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choline receptors (nAChRs) (14). Thus the relationship between the systemic nicotine-induced attenuation in the inflammatory response including pain and vascular changes and the PVN has been denoted.

The PSAR used in our study is introduced as a reversed passive Arthus reaction (15, 16). However, the inflammatory processes, such as the relationship between polymorphonuclear leukocyte (PMNs) movement and the chemical mediators relating to the plasma exudation, have not been studied. In this study, the inflammatory process of the PSAR was investigated to elucidate the mechanism whereby nicotine-induced endogenous glucocorticoids suppress the plasma exudation.

Materials and Methods

Animals

Wistar male rats (6-week-old) were purchased from Charles River Japan, Inc., Osaka, and Japanese white rabbits weighing about 3 kg were purchased from Kiwa Laboratory Institute, Wakayama and subjected to investigation after housing for at least 7 days. Animals were housed under constant temperature and humidity ($22 \pm 1^\circ\text{C}$, $55 \pm 10\%$) and with a fixed 12-h light-dark cycle and fed laboratory chow and water ad libitum. This animal study was carried out under the control of our committee in accordance with The Guidelines for Animal Experiments in Nara Medical University and Guiding principles for the care and use of laboratory animals approved by The Japanese Pharmacological Society.

Drugs and dosing

Wistar male rats received an intraperitoneal injection of superoxide dismutase (SOD, 52500 units/kg; Wako Pure Chemical Industries Ltd., Osaka) in saline solution 60 min before elicitation. *N*^G-Nitro-L-arginine-methyl ester (L-NAME, 100 mg/kg; Wako) or L-arginine (300 mg/kg; Nacalai Tesque, Kyoto) was intravenously injected just at the time of antigen challenge. Polyclonal rabbit anti-rat TNF- α antibody (5 μg /site; Innogenetics N.V., Gent, Belgium) was injected into the sensitized site at a volume of 0.05 ml at sensitization or just at elicitation. The animals received an intraperitoneal injection of nicotine tartrate dihydrate (0.8 mg/kg as nicotine, Nacalai Tesque) in a saline solution between 12:00 and 14:00. Mifepristone (30 mg/kg; Sigma, St. Louis, MO, USA) was subcutaneously injected 2.5 h before the nicotine injection. Mifepristone was suspended in peanut oil and this dose was selected to antagonize glucocorticoid receptors as reported elsewhere (17).

PSAR

Rabbit antiserum against ovalbumin (Sigma) was prepared as previously described (16). The titer of anti-serum was 1:8000, as estimated by the passive cutaneous anaphylaxis test with a 4-h latent period in Hartley male guinea pigs weighing 400–450 g. The anti-serum diluted from 1:4 to 1:16 with saline was injected intradermally at 0.05 ml/site onto the shaved back of male Wistar rats weighing 240–310 g. Four hours later, 0.5% Evans blue saline containing ovalbumin (OA, 2 mg/ml) was injected intravenously at 2.5 ml/kg to induce the PSAR. The animals were killed 1 h after antigen challenge. A 16-mm diameter punch of the skin from the Arthus lesion was used to measure the amount of dye according to the method of Harada et al. (18). This was subtracted by the value from the non-sensitized site to obtain the net value.

Myeloperoxidase (MPO) assay

The MPO assay was an adaptation of the method described by Bradley et al. (19). The MPO was extracted from homogenized tissue by suspending the material in 0.5% hexadecyl trimethylammonium bromide (Sigma) in 50 mM potassium phosphate buffer, pH 6.0. The specimens were freeze-thawed 3 times. Suspensions were then centrifuged at 3000 rpm for 10 min at room temperature, and the supernatant fraction was used for MPO activity determination. A 100- μl aliquot of supernatant was mixed with 2.90 ml of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml *o*-dianisidine dihydrochloride (Sigma) and 0.0005% hydrogen peroxide (Nacalai Tesque). Incubation was performed at room temperature for 10 min, and then the change in absorbance at 460 nm was measured with a spectrophotometer. The value from the non-sensitized site was subtracted from that of the sensitized site to obtain the net value.

Nitric oxide (NO) production by peritoneal exudate leukocytes

Peritoneal exudate leukocytes were obtained from Wistar male rats decapitated 4 h after an intraperitoneal injection with 10 ml of 1% (v/v) oyster glycogen in PBS (20). Peritoneal leukocytes were harvested following an intraperitoneal injection of Ca^{2+} -free HBSS (10 ml) containing 10 units/ml heparin. This cell suspension was centrifuged at $200 \times g$ for 10 min. The packed cells were rinsed three times and resuspended at a density of 3×10^7 cells/ml in HBSS containing 100 units/ml catalase and 330 units/ml SOD (21). After 1 h of incubation at 37°C in a water bath, the reaction was stopped by immersing the tubes in ice and cold centrifugation. The supernatant nitrite/nitrate was

determined by nitrite/nitrate Assay Kit-C II (Dojindo Lab., Kumamoto). The majority of cells were polymorphonuclear leukocytes.

Statistical analyses

Data were presented as the mean $\pm$ S.E.M. Statistical analysis was performed with one-way analysis of variance (ANOVA). If the group values were statistically significant, post hoc analyses were conducted using Scheffé test. Comparison of two different groups used the unpaired Student's *t*-test. *P* values of less than 0.05 were considered to indicate statistical significance.

Results

Mediators involved in PSAR

The PMNs infiltration as measured by extractable MPO activity was determined just before as well as after antigen challenge (Fig. 1). In addition, the contribution

Fig. 1. Neutrophil infiltration as measured by myeloperoxidase activity in the passive skin Arthus reaction just before and after antigen challenge. Each value indicates the mean $\pm$ S.E.M. for seven animals.

of pro-inflammatory cytokines were indicated on the basis of a significant inhibitory action on the dye leakage by a local injection with anti-rat TNF- α antibody at sensitization (Table 1).

Pretreatment with SOD (52500 unit/kg) 60 min before elicitation or L-NAME (100 mg/kg) just at elicitation significantly suppressed the dye leakage by 38% or 63%, respectively (Fig. 2). An intraperitoneal injection of SOD at this interval resulted in a more sustained effective blood level (22) and was sufficient to inhibit the early phase of immune-complex-induced injury (23, 24). In addition, the L-NAME dose suppressed NO-induced oedema (25, 26). Therefore these results indicate that production of both superoxide and NO in PMNs primed with TNF- α may contribute to the plasma exudation in the PSAR.

Effect of L-arginine on the nicotine-induced decreasing effect on the dye leakage

An intraperitoneal preinjection of nicotine (0.8 mg/kg) 30 min before elicitation suppressed the dye leakage by 54% similar to our previous report (14) (Fig. 3A) and was less effective on the PMNs infiltration (Fig. 3B). In addition, this nicotine-induced decreasing effect was substantiated by an intravenous injection with L-arginine (300 mg/kg) just at elicitation, which did not enhance the plasma exudation in the control group (Fig. 4). It seems that the nicotine-induced decreasing effect on the PSAR might be related to NO production in PMNs infiltrated at pre-challenge.

Effects of nicotine on NO production in peritoneum exudate PMNs

The ex vivo study was performed in order to elucidate the effect of nicotine on NO₂⁻/NO₃⁻ level released from PMNs primed with cytokine using peritoneal exudate leukocytes. Non-stimulated peritoneal exudate PMNs generated 10.6 μ mol/l of NO₂⁻/NO₃⁻. An intravenous injection of nicotine (0.8 mg/kg) 30 min before harvesting significantly inhibited NO₂⁻/NO₃⁻ level generation by 55% (Fig. 5A). In addition, this inhibitory action

Table 1. Effect of anti-rat TNF- α antibody on the passive skin Arthus reaction

Timing of treatment	Compound	No. of animals	Amount of dye (μ g/site)
At sensitization	saline	7	4.4 $\pm$ 0.94
	anti-rat TNF- α antibody	7	2.8 $\pm$ 0.66**
Just before challenge	saline	3	6.3 $\pm$ 0.71
	anti-rat TNF- α antibody	3	5.8 $\pm$ 0.39

Animals received an intradermal injection with saline or anti-rat TNF- α antibody (5 μ g/site) at sensitization or just before antigen challenge. Dye-leakage was measured 60 min after elicitation. Each point indicates the mean $\pm$ S.E.M. ***P* < 0.01: significantly different relative to the control, saline alone, by unpaired Student's *t*-test.

Fig. 2. Effects of superoxide dismutase and L-NAME on the dye leakage in the passive skin Arthus reaction. Animals received an intraperitoneal injection of saline or superoxide dismutase (SOD, 52,500 units/kg) 60 min before elicitation (A) or an intravenous injection of L-NAME (100 mg/kg) just before elicitation (B). Each point indicates the mean $\pm$ S.E.M. for five to four animals. * P <0.05, ** P <0.01: significantly different relative to the control, saline alone, by Student's t -test.

Fig. 3. Effect of nicotine on the dye leakage and neutrophil infiltration in the passive skin Arthus reaction. A: Dye-leakage of the passive skin Arthus reaction 30 min after saline or nicotine (0.8 mg/kg, i.p.). Each point indicates the mean $\pm$ S.E.M. for five animals. B: Myeloperoxidase activity just before and 1 h after elicitation in the passive skin Arthus reaction in animals treated with saline or nicotine. Each point indicates the mean $\pm$ S.E.M. for six animals. ** P <0.01: significantly different relative to the control, saline alone, by Student's t -test.

was abolished in animals pretreated with mifepristone (Fig. 5B). These results indicate that nicotine-induced elevation of blood corticosterone level may reflect the inhibitory effects on NO production in the PSAR.

Discussion

Previously, we reported that a single systematic

administration of nicotine inhibited the plasma exudation in the PSAR in rats by elevation of blood corticosterone levels (14). The steroid elevation is mediated by brain nAChRs as demonstrated through use of PVN-lesioned animals, mecamylamine, and the glucocorticoid receptor antagonist mifepristone. The results presented here demonstrate that nicotine-induced endogenous corticosterone may inhibit the plasma exudation

Fig. 4. Effect of L-arginine on nicotine-induced inhibitory action in the passive skin Arthus reaction. Animals received an intraperitoneal injection of saline or nicotine (0.8 mg/kg) 30 min before elicitation and an intravenous injection of saline or L-arginine (300 mg/kg) just before elicitation. Each point indicates the mean $\pm$ S.E.M. for five to four animals. ** $P < 0.01$: significantly different relative to the control, saline alone, by ANOVA.

by suppressing nitric oxide production in PMNs primed with TNF- α .

The Arthus reaction is an acute inflammatory response resulting from deposition of immune complexes with tissue. The formation of immune complexes

induces the activation of the complement system. Complement activation products, such as C3a and C5a, play a major role in edema formation causing PMNs infiltration, prostaglandin production, histamine release from mast cells, and finally, the PMNs release of several mediators, for example, lysosomal enzymes during phagocytosis of immune complexes. The Arthus reaction is elicited as a reverse passive phenomenon in which the antibody is injected at the reaction site and the antigen is introduced intravenously. In general, the reverse passive Arthus reaction (RPA) model is used to evaluate anti-inflammatory compounds. The plasma exudation and PMNs infiltration serve as indexes for evaluating the anti-inflammatory activities. In the RPA, during phagocytosis of immune complexes or following exposure to chemotactic factors, the PMNs release oxygen-derived free radicals such as superoxide anion (23, 24, 27) and then generate nitric oxide as a second major oxidant-generating pathway via inducible nitric oxide synthase, which is transcriptionally up-regulated by pro-inflammatory cytokines such as TNF- α , reacting with L-arginine (26, 28). These factors from PMNs also cause the increase of plasma exudation.

The PSAR used in our study has been introduced as the RPA in rat skin, which shows the intensive plasma exudation by an intravenous injection with OA saline solution at 4 h after local injection with anti-OA rabbit

Fig. 5. Effect of nicotine on nitric oxide production in peritoneal exudate neutrophils in rats. The peritoneal exudate neutrophils were harvested 4 h after an intraperitoneal injection of oyster glycogen. Cells were cultured for 1 h, and nitric oxide production was analyzed by measurement of nitrite in culture supernatants. A: Animals received saline or nicotine (0.8 mg/kg, i.v.) 30 min before harvesting. Each point indicates the mean $\pm$ S.E.M. for six animals. B: Mifepristone (30 mg/kg) was subcutaneously injected 3 h before harvesting in animals treated with nicotine or saline. Each point indicates the mean $\pm$ S.E.M. for four to five animals. * $P < 0.05$: significantly different relative to the control, saline alone, by Student's *t*-test.

serum, and is characterized by its independence from mast cell degranulation and sensitivity to prednisolone but not indomethacin as compared with 48- and 5-h homologous passive cutaneous anaphylaxis in rats (15), carrageenin-induced paw edema, and adjuvant-induced arthritis in rats (16). This evidence indicates that prostaglandin and histamine (as inflammatory mediators for vascular permeability) are not responsible for the PSAR. From this evidence, it appears that sensitization may induce some event during pre-challenge which may play an important role in the plasma exudation in the PSAR. In this regard, we found that PMNs infiltrate just before as well as after elicitation, suggesting induction of non-specific acute inflammation may be due to a local injection with anti-OA rabbit serum. In addition, the induction of pro-inflammatory cytokine, $\text{TNF-}\alpha$, during pre-challenge was determined on the basis of the inhibitory action of a local injection of anti- $\text{TNF-}\alpha$ antibody on the plasma exudation. Warren (28) reported that locally administered anti- $\text{TNF-}\alpha$ antibody was ineffective on vasopermeability changes and neutrophil recruitment of the RPA in rats. Thus our results indicate that anti- $\text{TNF-}\alpha$ antibody may neutralize endogenous $\text{TNF-}\alpha$ produced during the pre-challenge, an effect not shown with anti-OA rabbit. From these results, it seems that acute inflammatory phenomena including $\text{TNF-}\alpha$ and pre-infiltrated PMNs may be important in the PSAR, and the anti-inflammatory actions of glucocorticoids on prostaglandin synthesis (29, 30) and histamine-induced vascular permeability (31, 32) may be independent of the nicotine-induced decreasing effect on the plasma exudation in the PSAR.

Pro-inflammatory cytokines, such as $\text{TNF-}\alpha$ and $\text{INF-}\gamma$, are reported to be important factors enhancing the inflammation following the priming effects on PMNs functions such as superoxide anion production (33) and NO generation consequent to induction of nitric oxide synthase (iNOS) (20). Some studies reported that NO is generated by expression of iNOS in proportion to PMNs accumulation in the acute inflammatory response observed during zymosan-activated plasma-induced paw inflammation (34), zymosan-mediated cutaneous inflammation (35), and carrageenan-induced paw inflammation (26). Therefore, we questioned whether superoxide and/or NO generation reflect the plasma exudation of the PSAR. We observed that both nitric oxide and superoxide anion were responsible for the plasma exudation in the PSAR using pretreatment with SOD or L-NAME. The interaction of superoxide and NO can yield secondary products that may be more reactive or cytotoxic, specifically, peroxynitrite (ONOO^-), which is injurious itself as well as serving as a precursor for other reactive species, resulting in

tissue injury (36). In addition, Mulligan et al. showed that the production of NO under the condition of superoxide production could enhance the vascular permeability during the late phase in the RPA in rat skin and that protective doses of the L-arginine analog N^G -monomethyl-L-arginine did not interfere with recruitment of PMNs in immune complex-induced lung injury (37). Therefore, it is possible that pre-infiltrated PMNs primed with $\text{TNF-}\alpha$ may play an important role in the plasma exudation of the PSAR via both superoxide and NO production.

From these results, we assessed the influence of a systematic pre-administration of nicotine on the PMNs filtrate and NO production. Pretreatment with nicotine was less effective on the PMNs infiltration just before and after elicitation in contrast to nicotine's decreasing effect on the plasma exudation. On the other hand, we observed that intravenous supplementation with L-arginine just at elicitation reversed the nicotine-induced decreasing effect without enhancing the plasma exudation in the control group. Additionally, an intravenous injection of nicotine before harvesting suppressed $\text{NO}_2^-/\text{NO}_3^-$ levels released from PMNs harvested at 4 h after an intraperitoneal injection with oyster glycogen. Moreover, this systematic inhibitory action of nicotine on NO production in the peritoneal exudate PMNs was substantiated in animals pre-treated with mifepristone. This antagonistic action of mifepristone correspond to its inhibitory action on nicotine-induced decreasing effect on the plasma exudate in our previous study. The peritoneal exudate PMNs at 4 to 6 h after intraperitoneal injection with oyster glycogen generate NO via iNOS induced by in vivo and ex vivo stimulation with $\text{TNF-}\alpha$ and inflame (20). Extracellular L-arginine could be taken up and utilized for NO synthesis by PMNs after LPS treatment but not non-stimulated PMNs (38). Therefore, it is suggested that nicotine exerts its anti-inflammatory activity indirectly on NO generation by increasing blood corticosterone levels. A high dose of L-NAME (100 mg/kg) inhibited 65% of the PMN infiltration after elicitation, while in preliminary studies, neither $\text{TNF-}\alpha$ or SOD exerted a significant effect (data not shown). Although further studies are needed to clarify the relationship between the inhibitory mechanism of L-NAME on the PMN infiltration and the plasma exudation in the PSAR, it appears that pre-filtrated PMNs rather than post-filtrated PMNs may be an important factor for the plasma exudation of the PSAR.

The mechanism(s) of this inhibitory action of glucocorticoid on NO production in PMNs primed with cytokines was not clear in this study. However, the inhibitory action of glucocorticoid is different from an inhibitory action of dexamethasone on NO produc-

tion. Dexamethasone suppresses iNOS expression in the peritoneal exudate PMNs in presence of L-arginine (20) resulting from the reversible effect of L-arginine on the nicotine-induced decreasing effect on the plasma exudation. Yang et al. reported that dexamethasone inhibits NO production without decreasing iNOS expression via lipocortin 1, an anti-inflammatory peptide induced by glucocorticoid in adjuvant arthritis synovial macrophages (39). Considering that immunological inflammation such as the Arthus reaction could initiate and/or promote chronic inflammatory disorders including rheumatoid arthritis and that the suppression of the immunological inflammations by anti-inflammatory steroids plays an important role in their clinical effect (40–45), lipocortin 1 might be responsible for the anti-inflammatory action of nicotine-induced release of endogenous corticosterone on the PSAR. The mechanism(s) of inhibitory action of endogenous corticosterone on the NO production in PMNs primed with cytokines requires further investigation.

In conclusion, the plasma exudation of the PSAR, which was elicited 4 h after skin was sensitized with antiserum, was dependent on the generation of superoxide anion and nitric oxide from PMNs primed with cytokines such as TNF- α . The nicotine-induced decreasing effect on the plasma exudation in this model was not dependent on PMN infiltration and was reversed in animals supplied with L-arginine. Systemic administration of nicotine inhibited NO production from peritoneal exudate PMNs, which was demonstrated by pretreatment with a glucocorticoid receptor antagonist. Therefore these results indicate that a systematic administration of nicotine might inhibit the plasma exudation of the PSAR through suppressing NO production in PMNs primed with cytokines via elevating endogenous corticosterone levels.

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