Role of Prostaglandin E₂ and Leukotriene B₄ in Skin Reaction Induced by Transdermal Application of Propranolol

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Dermal application of propranolol (PRL) induced formation of erythema and edema, and pseudoeosinophil infiltration in epidermis and dermis at the application site in guinea pigs. We investigated the production of prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄) at the application site of PRL and the role of these inflammatory chemical mediators in the occurrence of the skin reactions. PGE₂ was found to be produced at the application site slightly after the accumulation of PRL released from the adhesive bandage in the patch test, and the amount of PGE₂ increased continuously, with a peak value obtained at 24 h after application. The time-course changes resembled those of Δa^* value, the index of erythema formation determined by colorimetric measurement, and edema formation. The production of PGE₂ by dermal application of PRL was suppressed by local pre-treatment with dexamethasone or indomethacin. However, no notable production of LTB₄ was observed at the application site of PRL.

Key words propranolol; prostaglandin E₂; leukotriene B₄; erythema; edema; dermal application

Transdermal delivery of propranolol (PRL), which is used for angina pectoris and other cardiac diseases, has several merits since it rules out the hepatic first pass effect and improves patient compliance. Krishna *et al.*¹⁾ reported that transdermal application of the patch formulation of PRL-HCl to the inner pinna of rabbits, induced a 20-fold higher bioavailability compared to that obtained by oral administration. However, they noticed mild skin irritation by dermal application of the patch.²⁾ When PRL was applied transdermally to the flank of guinea pig, very slight to well defined erythema was recognized at a 2.5% concentration of PRL, well defined erythema was recognized at a 5% concentration, and moderate erythema was recognized with a 10% concentration.³⁾ Further, we reported that the development of skin irritation reactions of β -blockers having various kinds of aromatic ring structures was caused mainly by the side chain rather than the aromatic ring of the drugs.⁴⁾ A primary skin reaction to the irritation of a dermal formulation is a vascular response to microcirculation, and several inflammatory chemical mediators such as histamine, bradykinin and/or arachidonic acid metabolites play role in this process. We have found that dexamethasone (DEX) and indomethacin (INM) have suppressive effects on erythema formation induced by the dermal application of PRL in guinea pigs and suggested the participation of arachidonic acid metabolites. In addition, we reported pseudoeosinophil infiltration in the dermal application site in guinea pigs.³⁾ Amongst arachidonic acid metabolites, prostaglandin E₂ (PGE₂) increases the local blood flow by intradermal administration⁵⁾ and enhances the vascular permeability induced by other chemical mediators such as bradykinin.^{6–8)} Leukotriene B₄ (LTB₄) has polymorphonuclear leukocyte migration effects^{9,10)} and induced intraepidermal neutrophil microabscesses by topical application.¹¹⁾ In addition, intradermal injection of LTB₄ together with PGE₂ or bradykinin produced a significant increase in plasma exudation.¹²⁾ From these findings described above, it is suggested that these two chemical mediators may play a role in the skin reactions induced by dermal applica-

tion of PRL. However, there are few reports which clarify the role of inflammatory chemical mediators on skin reactions induced by dermal application of PRL. The objective of the present study is to assess the production of PGE₂ and LTB₄ at the application site of PRL and the roles of these mediators in the appearance of skin reaction induced by PRL, and to obtain information helpful to development of a transdermal drug delivery system with less irritation potential.

MATERIALS AND METHODS

Reagents *dl*-Propranolol hydrochloride (PRL-HCl), DEX and INM were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Propranolol base (PRL) was prepared from PRL-HCl by alkalization with sodium hydroxide according to the method described in the Japan Pharmacopoeia.

Animals Male Std:Hartley guinea pigs (clean) were purchased from Japan SLC, Inc. (Hamamatsu, Japan) at 4–5 weeks of age. After 11–18 d acclimation, animals weighing 308–522 g were used in the study. For assessment of production of PGE₂ and LTB₄, 4 animals were used for each application time of PRL. Suppressive effects of anti-inflammatory agents were examined using 8 animals for each anti-inflammatory agent, and 4 out of these 8 animals were used for measurement of PGE₂ and the remaining 4 animals were used for histopathological examination.

Administration of PRL Both side areas of the flank of guinea pigs were shaved. On one side, an adhesive bandage for patch test (Torii Pharmaceutical Co., Ltd., Tokyo, Japan, hereafter designated as adhesive bandage) which contained 0.1 ml of a 5% ethanolic solution of PRL was applied for 0, 2, 4, 8, 16, 24 or 48 h, and on the other side, an adhesive bandage which contained 0.1 ml of ethanol was applied as control. In the test to assess the effects of anti-inflammatory agents, 0.1 ml of a 5% ethanolic solution of PRL was applied on both sides for 24 h.

Application of Anti-inflammatory Agents One hour

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Table 1. Evaluation of Edema Formation

No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (area raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure)	4
Total possible edema score	4

prior to application of PRL, 20 μ l of propylene glycol: ethanol (1:1) solution (hereafter designated as propylene glycol/ethanol) containing INM or DEX at a concentration of 3% (w/v), the concentration which manifested the anti-inflammatory effect clearly,⁴⁾ was applied at an area 20 mm diameter on one side, and the other side was treated similarly with 20 μ l of propylene glycol/ethanol only.

Assessment of Skin Reaction Prior to application of PRL or anti-inflammatory agents and after application of PRL, redness of skin (α^* value) was measured using a colorimeter (Chroma Meter CR-200, Minolta, Osaka, Japan). An increase of redness was expressed as differences of α^* values ($\Delta\alpha^*$) prior to application of PRL or anti-inflammatory agents, and after application of PRL. Edema formation was scored after application of PRL according to the criteria of Draize *et al.*¹³⁾ as shown in Table 1.

Quantitative Analysis of PGE₂ and LTB₄ Guinea pigs were sacrificed by exsanguination after a defined application time of the adhesive bandage which contained 0.1 ml of a 5% ethanolic solution of PRL. Skin of 16 mm diameter was taken and frozen in 5 ml of 95% ethanol cooled by acetone-dry ice, and then cut into small pieces. Extraction of PGE₂ and LTB₄ was carried out according to the method described by Inoue *et al.*¹⁴⁾ The pieces of skin were homogenized in 95% ethanol cooled by acetone-dry ice using a high speed homogenizer (Physoctron NS-50; NITI-ON, Chiba, Japan), and then 15 ml of ethanol was added to the homogenate and mixed completely. The homogenate was centrifuged for 10 min at 4°C and 12000 $\times g$. Nine ml of the supernatant was transferred into 2 silicon pre-treated flasks, and then concentrated to approximately 0.5 ml of volume in a vacuum. These samples were purified as shown below. A sample for assay of PGE₂ was supplemented with 2 ml of methanol and centrifuged for 10 min at 4°C and 3000 $\times g$. The supernatant was collected and mixed with 16 ml of ultrapure water. The pH of this solution was adjusted to 4 with 0.1 N hydrochloric acid, and then applied on to Sep-Pak C₁₈ column (Waters, MA, U.S.A.) which was washed in advance with 5 ml of methanol and then with 5 ml of ultrapure water. The column was washed with 5 ml of ultrapure water followed by 5 ml of hexane, and then the PGE₂ was eluted with 5 ml of ethylacetate containing 1% methanol. The eluate was dried up and dissolved in 1 ml of enzyme immunoassay (EIA) buffer solution, and the amount of PGE₂ was quantified using a PGE₂ EIA kit (Cayman). For assay of LTB₄, the sample was supplemented with 2 ml of methanol and centrifuged for 10 min at 4°C and 1500 $\times g$. The supernatant was collected and mixed with 8 ml of 0.1 M phosphate buffer solution (pH 4, PBS), and then applied on to Sep-Pak C₁₈ column which was washed in advance with 5 ml of ethanol and then with 5 ml of ultrapure water. The column was washed with 5 ml of ultra-

Fig. 1. Time-Course Change of Cumulative Amount of PRL Released from an Adhesive Bandage

The cumulative amount of PRL released from an adhesive bandage tape was calculated from the difference between the amount of PRL mounted on the tape and the amount remaining after application. Each circle and bar represent the mean $\pm$ S.E. of 4 animals.

pure water followed by 5 ml of hexane, and then the LTB₄ was eluted with 5 ml of a mixed solution of ethanol and ultrapure water (90:10). The eluate was dried up and dissolved in 1 ml of EIA buffer solution, and the amount of LTB₄ was quantified using a LTB₄ EIA kit (Cayman).

Histopathological Examination The skin, to which was applied PRL after pre-treatment with anti-inflammatory agents or propylene glycol/ethanol, was subjected to the routine process of fixation in a formalin solution, embedding in paraffin, staining with hematoxylin-eosin and histopathological examination. The examination was subcontracted to Nippon Experimental Medical Research Institute Co., Ltd. (Gunma, Japan).

Quantitative Assay of PRL The remaining PRL in the adhesive bandage after application was extracted with methanol and assayed by a HPLC method under the conditions described in the previous report.¹⁵⁾ The cumulative amount released from the adhesive bandage was calculated from the differences between the amount of PRL mounted on the adhesive bandage and the amount remaining after application.

Statistical Analysis $\Delta\alpha^*$ values in the groups treated with PRL and ethanol were analyzed by Student's *t*-test.

RESULTS

Release of PRL The cumulative PRL amount released from the adhesive bandage at each defined application time is shown in Fig. 1. PRL was released from the adhesive bandage to the skin surface rapidly after application, and the cumulative amount showed a linear increase up to 48 h after initiation of application showing 0.59 mg/cm² at 4 h and 0.99 mg/cm² at 48 h.

Evaluation of Skin Reaction Time-course of $\Delta\alpha^*$ value and edema formation is shown in Fig. 2, and the suppressive effects of anti-inflammatory agents on the increase in $\Delta\alpha^*$ value are shown in Fig. 3. $\Delta\alpha^*$ value showed an apparent increase at 4 h as 4.2, and it increased time dependently up to 7.9 at 24 h. Edema formation was observed slightly during 8–24 h after application. An increase in $\Delta\alpha^*$ value induced

Fig. 2. Time-Course Change of Δa^* Value and Edema Formation Induced by PRL

Δa^* value was calculated from the difference prior to and after application of PRL. Closed circle means Δa^* value by PRL, and open circle means Δa^* value by ethanol. Closed triangle means edema formation by PRL application, and open triangle means edema formation by ethanol application. Each circle and bar for Δa^* value represents the mean $\pm$ S.E. of 4 animals, and each triangle for edema formation represents the mean of 4 animals. **: $p < 0.01$ vs. ethanol group. Data were evaluated by Student's *t*-test.

Fig. 3. Effects of Anti-inflammatory Agents on the Increase in Δa^* Value

Δa^* value was calculated from the differences between prior to and after application of PRL. Each column and bar for Δa^* value represents the mean $\pm$ S.E. of 8 animals in the presence and absence of pre-treatment with anti-inflammatory agents. **: $p < 0.01$ vs. PRL alone group. Data were evaluated by Student's *t*-test.

Fig. 4. Time-Course Change of PGE_2 Production Induced by PRL

Closed circle means PGE_2 production by PRL, and open circle means PGE_2 production by ethanol. Each circle and bar represents the mean $\pm$ S.E. of 4 animals. *: $p < 0.05$ vs. ethanol group. Data were evaluated by Student's *t*-test.

Fig. 5. Time-Course Change of LTB_4 Production Induced by PRL

Closed circle means LTB_4 production by PRL, and open circle means LTB_4 production by ethanol. Each circle and bar represents the mean $\pm$ S.E. of 4 animals. **: $p < 0.01$ vs. ethanol group. Data were evaluated by Student's *t*-test.

Fig. 6. Effects of Anti-inflammatory Agents on the Production of PGE_2

Each column and bar for PGE_2 production by PRL represent the mean $\pm$ S.E. of 4 animals in the presence and absence of pre-treatment with anti-inflammatory agents. **: $p < 0.01$ vs. PRL alone group. Data were evaluated by Student's *t*-test.

by dermal application of PRL was significantly suppressed by pre-treatment with DEX and INM. In the preliminary study, an increase in Δa^* value induced by dermal application of ethanol for 24 h was not significantly suppressed by pre-treatment with DEX and INM (inhibitory values of Δa^* : 1.26 ± 0.59 and 0.40 ± 0.30 (mean $\pm$ S.E., $n=8$), respectively).

Production of PGE_2 and LTB_4 Time-course of PGE_2 and LTB_4 production is shown in Figs. 4 and 5. Suppressive effects of anti-inflammatory agents on the production of PGE_2 by dermal application of PRL are shown in Fig. 6. Recovery rates of PGE_2 and LTB_4 from the skin were 95% and 60%, respectively. Production of PGE_2 was noticed at 4 h after application and increased thereafter until 24 h when the peak value was obtained. However, no apparent increase in LTB_4 was induced by dermal application of PRL. An increase in PGE_2 induced by application of PRL was significantly suppressed by pre-treatment with DEX and INM.

Histopathological Findings Table 2 shows the histopathological findings of the skin to which was applied PRL after pre-treatment with anti-inflammatory agents. At the regions pre-treated with DEX or INM, very slight or mild changes were observed including pseudo eosinophil infiltration in epidermal, degeneration/necrosis of epidermal and in-

Table 2. Histopathological Findings in Guinea Pigs Following Transdermal Application of PRL

Findings	Grade	Pre-treatment of anti-inflammatory drugs															
		INM								DEX							
		Non-tre.				Treated				Non-tre.				Treated			
		—	±	+	++	—	±	+	++	—	±	+	++	—	±	+	++
Epidermis																	
Pseudoeosinophil infiltration	2	1	1	0	0	2	1	0	0	2	1	0	1	3	1	0	0
Degeneration/necrosis	0	2	2	0	0	1	3	0	0	0	2	1	1	2	1	1	0
Hyperplasia	4	0	0	0	4	0	0	0	0	1	0	3	0	4	0	0	0
Dermis																	
Hemorrhage in shallow area	4	0	0	0	4	0	0	0	0	4	0	0	0	4	0	0	0
Mononuclear cell infiltration	1	2	1	0	0	2	2	0	0	0	3	1	0	0	4	0	0
Pseudoeosinophil infiltration	0	3	1	0	0	3	1	0	0	2	0	1	1	1	1	2	0

The grades of changes of histopathological findings are as follows: —, no marked change; ±, very slight changes; +, slight changes; ++, moderate changes. The numerical values in the table mean number of animals showing the corresponding findings.

filtration of mononuclear cell and pseudoeosinophil in dermal. However, similar changes were also observed at the sites without pre-treatment with anti-inflammatory agents.

DISCUSSION

Arachidonic acid metabolites play an important role as chemical mediators for the occurrence of acute inflammatory reactions. It has been reported that prostaglandins are produced in the skin after irradiation of UV-B,¹⁶⁾ and that development of increased prostaglandin E levels is parallel to the development of erythema formation.¹⁷⁾ Moreover, a carrageenin inflammation model revealed induction of prostaglandin E in the exudate suggesting a participation of PGE₂ for exudation.^{18,19)} It is also known that LTB₄ in the exudate by carrageenin inflammation correlates to chemotaxis and degranulation of polymorphonuclear leukocytes.²⁰⁾ From these findings, we speculated that PGE₂ and LTB₄ were involved in the expression of the skin response induced by PRL in guinea pigs. Accordingly, we investigated the production of PGE₂ and LTB₄ at the dermal application site of PRL and the possible interactions of PGE₂ and LTB₄ with the occurrence of inflammatory skin reactions.

Dermal application of PRL induced the production of PGE₂ at the application site. Production of PGE₂ increased time-dependently and the time-course change was similar to those of erythema and edema formation. The synchronism of PGE₂ production and erythema formation has been reported for retinoic acid (systemic vitamin A acid) in guinea pigs.²¹⁾ We previously reported that pseudoeosinophiles infiltration in dermis and epidermis and mononuclear cells infiltration in dermis were observed at 8 h of PRL application, and the histological changes were aggravated at 16 h of application.⁴⁾ In this study, PGE₂ production was noticed at 4 h after application and increased thereafter until 24 h when the peak value was obtained. These results suggest that pseudoeosinophiles and mononuclear cells are concerned in the PGE₂ production induced by PRL. Production of PGE₂ by PRL was suppressed by pre-treatment with DEX and INM. INM is a non-specific inhibitor of cyclooxygenase I/cyclooxygenase II, and DEX is reported to suppress the induction of cyclooxygenase II.²²⁾ These results suggest that cyclooxygenase II may be induced by the dermal application of PRL.

Contrary to this, no induction of LTB₄ was noticed at the application site of PRL. In addition, pre-treatment with DEX showed no significant suppressive effect on pseudoeosinophil infiltration induced by PRL. Since LTB₄ was not found in the exudate of the carrageenin pleuritic model of rats,²³⁾ it suggested the possibility that LTB₄ was not involved in acute skin reactions induced by dermal application of PRL. It has been reported that PGE₂ alone induces no leukocyte infiltration, however, it enhances neutrophil infiltration induced by zymosan-activated plasma.²⁴⁾ Therefore, it is considered that prostaglandins may indirectly relate to pseudoeosinophil infiltration induced by PRL.

As described above, these results suggest that PGE₂ induced by dermal application of PRL participated in erythema and edema formation at the skin. However, no induction of LTB₄ was observed. For the development of less irritating transdermal drug delivery systems for PRL, we consider that one of the preferable approaches is to suppress the production of prostaglandin by control of transdermal drug absorption. Pseudoeosinophil infiltration induced by dermal application of PRL suggests the necessity for further investigations concerning the effects of PRL on leukocytes and other participant cells.

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