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Report

Effects of Dithiothreitol and Ascorbate on the Penetration of Diclofenac Across Excised Rat Dorsal Skin

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The penetration of diclofenac through excised rat dorsal skin was found to be poor. Treatment of the skin with a reductant such as dithiothreitol or ascorbate increased the permeability of the skin to diclofenac, along with an increase in the protein thiol content of the stripped skin surface tissue. An increase in protein thiol content may increase the hydration capability of the stratum corneum.

KEY WORDS: transdermal penetration; rat dorsal skin; sodium diclofenac; antioxidants; protein thiol.

INTRODUCTION

There have been many attempts to enhance transdermal absorption of drugs (1–5). Since some organic solvents have been found to enhance transdermal absorption of drugs by disordering the lipid layer of the stratum corneum, it has been proposed that an ordered matrix of intercellular lipid plays a major role in preventing transdermal penetration of various compounds (6,7). Surfactants increase the permeability of the stratum corneum, and the suggested mechanisms involve not only the disordering of the lipid layer but also denaturation of keratin in the stratum corneum (3). Since major constituents of the stratum corneum are proteins (8), the role of protein in the barrier function of stratum corneum is of interest.

In the present study, we investigated the effects of reductants such as dithiothreitol (DTT) and ascorbate on the permeation of diclofenac through excised rat dorsal skin *in vitro*.

MATERIALS AND METHODS

Materials

DTT, sodium ascorbate, and sodium diclofenac were obtained from Sigma Inc. (St Louis, Mo.). Other reagents used were of analytical grade.

In Vitro Penetration Study

Wistar male rats weighing 200 to 250 g were used. The dorsal hair of the rats was shaved with an electric clipper and the dorsal skin tissue excised, as described previously (9). The excised skin included subcutaneous tissue.

The *in vitro* penetration study was performed using an LG-1084-LPC penetration cell (Laboratory Glassware Inc., Berkeley, Calif.) according to the method described previously (9). Briefly, 5.5 ml of a 0.05 *M* isotonic phosphate buffer solution (pH 7.4) was maintained at 32°C on the receptor side (subcutaneous tissue side) and 2 ml of the buffer solution containing 2.5 mg of sodium diclofenac was placed in the donor side (stratum corneum side). Following the application of the test solution in the donor side, 100-μl aliquots from the receptor side were collected at 2-hr intervals for 12hr. After each sampling, 100 μl of the isotonic buffer solution was added. The surface area of the skin membrane exposed to the solution was 531 mm^2 (26-mm-diameter circle).

After the penetration study, the skin surface, including the stratum corneum and possibly the epidermis, was separated by stripping (since stripping of the surface tissue was difficult immediately after excision, control skin surface tissue was obtained after immersion in the isotonic buffer for 12hr). After homogenization of the stripped skin surface tissue, the protein thiol content was measured.

Assay

Assay of protein thiol was performed by the method reported by Di Monte *et al.* (10), with glutathione as the standard thiol. The protein assay was performed by the method of Lowry *et al.* (11), using bovine serum albumin as standard. Diclofenac was assayed by high-performance liquid chromatography (12).

RESULTS

The penetration of diclofenac through excised rat dorsal skin occurred slowly. Diclofenac penetration (shown as the solid line in Fig. 1) was observed to be linear after a lag time of about 4 hr (Table I). At steady state, the apparent pene-

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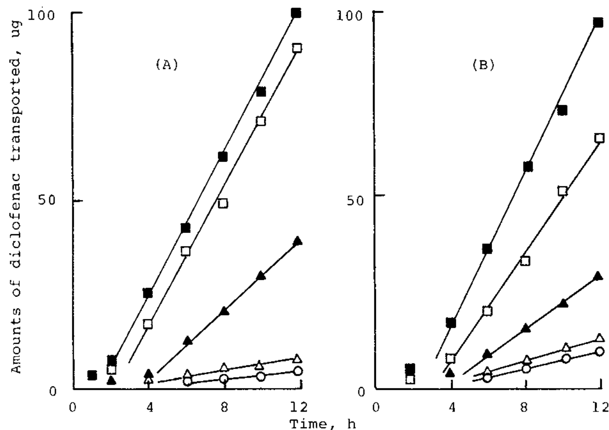

Fig. 1. Effect of dithiothreitol (DTT) (A) and ascorbate (B) on the transport of diclofenac through the excised rat dorsal skin including subcutaneous tissue. Transport of diclofenac was assessed as the amount of diclofenac transported into the receptor medium when 4 ml of a solution containing 1.25 mg of diclofenac/ml was placed on the donor side. Symbols represent the following additive concentrations: ○, no additive; △, 1.25 m*M*; ▲, 6.25 mM; □, 12.5 m*M*; ■, 25 mM. Each value represents the mean ± SD (N = 4 to 6).

tration rate constant, k_{ss}, was determined using the following equation:

$$k_{ss} = (\Delta Q/\Delta t)/[(\text{area}) \times (\text{initial concentration on the donor})]$$

where $\Delta Q/\Delta t$ is the mass transported across the membrane per unit time. Diclofenac penetration was accelerated by the addition of DTT to the applied solution on the donor side. The penetration rate constant of diclofenac at steady state increased with increases in the concentration of DTT up to 12.5 m*M* (Table I). The rate constant and penetration amounts of diclofenac in the presence of 25 m*M* DTT in the donor side were approximately 20 times greater than those observed in the absence of DTT (Table I).

The presence of ascorbate in the applied solution also accelerated diclofenac penetration across the excised skin. Penetration increased with higher ascorbate concentrations (Fig. 1). The penetration rate constant of diclofenac in the presence of 25 m*M* ascorbate was approximately 10 times greater than that found in the absence of any additive (Table I). An increase in the concentration of either DTT or ascorbate resulted in a reduction of the lag time for diclofenac transport (Table I).

The protein thiol content in the stripped dorsal skin surface tissue was increased by treatment of either DTT or ascorbate (Table I). DTT, 12.5 and 25 m*M*, increased the protein thiol content twofold. As shown in Fig. 2, a plot of the penetration rate of diclofenac against the protein thiol content gave a sigmoidal pattern.

DISCUSSION

In vitro penetration of diclofenac across excised rat dorsal skin is poor (9). The rate-limiting step for diclofenac penetration was found to be the transport of diclofenac through the stratum corneum. In the present study, diclofenac penetration was less than 0.2% over 12hr.

The presence of the antioxidants, DTT and ascorbate, increased the penetration of diclofenac markedly. A parallel increase in the protein thiol content in the stripped dorsal skin surface (probably including both stratum corneum and epidermis) was also observed.

It has been proposed that the poor permeability of the stratum corneum of the skin is due to an ordered matrix of intercellular lipid and to the low content of water (13). Further, protein represents a major constituent of the stratum corneum, and 90% of proteins are water-insoluble protein (3). The protein thiol contents in hepatocytes (14) and intestinal epithelial cells (15) are about 100 and 30 μmol/g protein, respectively, and the protein thiol contents of intestinal tissue (15) and subcutaneous tissue (unpublished data) 100 and 60 μmol/g protein, respectively, while the protein thiol content in the stripped rat dorsal skin surface tissue was found to be low, i.e., about 6 μmol/g protein. A reduction of

Table I. Effect of Dithiothreitol (DTT) and Ascorbate on the Penetration of Diclofenac Through Excised Rat Dorsal Skin Including Subcutaneous Tissue and on the Protein Thiol Content in Stripped Rat Dorsal Skin Surface Tissue (Without Subcutaneous Tissue)

Expt No.	Additive and conc.	k_{ss} (μg · hr/cm²)	Lag time (hr)	Protein thiol content (μmol/g protein)
0	Control, no additive	0.47 ± 0.19	3.9 ± 0.3	6.1 ± 1.2
	DTT			
1	1.25 m*M*	0.98 ± 0.27*	3.4 ± 0.5	6.7 ± 1.7
2	6.25 m*M*	4.22 ± 0.96**	3.3 ± 0.3	8.9 ± 1.4*
3	12.5 m*M*	9.12 ± 2.4**	2.2 ± 0.2**	11.9 ± 2.9**
4	25 m*M*	9.24 ± 1.91**	1.3 ± 0.2**	13.9 ± 1.7**
	Ascorbate			
5	1.25 m*M*	0.57 ± 0.21	3.7 ± 0.2	6.0 ± 1.4
6	6.25 m*M*	1.81 ± 0.42*	3.5 ± 0.3	7.4 ± 1.7
7	12.5 m*M*	3.88 ± 0.74**	3.2 ± 0.3	8.0 ± 1.2
8	25 m*M*	5.09 ± 1.64**	2.6 ± 0.4	9.5 ± 1.9*

[a] Penetration rate constant of diclofenac at steady state. Each value represents the mean ±SD (N = 4 to 6).

* $P < 0.05$ versus control.

** $P < 0.01$ versus control (Student's t test).

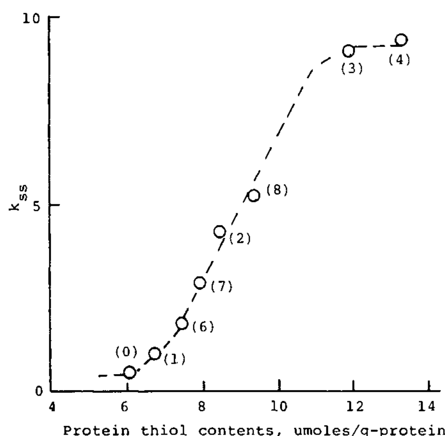

Fig. 2. Relationship between the penetration of diclofenac through the skin and the protein thiol content in the stripped skin surface tissue, as obtained from Table I. The numbers by each symbol represent the experimental number described in Table I.

the protein thiol content in rat intestinal tissue reduced the permeation of various drugs through intestinal tissue (15). Thus, it is suggested that DTT and ascorbate increase the penetration of diclofenac across excised rat dorsal skin by increasing the protein thiol content in the skin surface. It has been reported that protein in keratinized tissue is rich in cysteine residues (16), and its insoluble nature might be attributed to strong chemical bonds such as disulfide bridges (17). In fact, a strong reducing agent such as 2-mercaptoethanol that cleaves disulfide bonds results in hydration and further solubilization of proteins in keratinized tissue (18). Since the increase in the protein thiol content may represent a reduction of the number of disulfide bridges in the protein, the hydration capability of the protein may increase along with an increase in the protein thiol content.

The low permeability of stratum corneum may be due partly to the low content of protein thiols in the stratum corneum, resulting in a low propensity for hydration of the tissue. Occlusion of the skin increased the permeability of the stratum corneum along with an increase in hydration of the tissue (19). Therefore, the increase in the permeability of the stratum corneum by treatment with a reducing agent (antioxidant) may be due to the increase in hydration of the stratum corneum. However, since diclofenac skin penetration reached only 4% over 12hr even in the presence of 25 m*M* DTT, intercellular lipid of the stratum corneum rather than protein of the stratum corneum may play the major role as a penetration barrier of exogenous compounds.

In the present study, we did not measure the penetration of either DTT or ascorbate across the skin. Thus, it is not clear whether the observed linear nature (steady state) of diclofenac transport in the presence of antioxidants in the donor phase involves the rapid transport of the antioxidant, which would reduce the concentration in the donor to less than an effective concentration. However, if transport of the antioxidant from the donor to the receptor occurred rapidly, the effectiveness of either antioxidant should gradually decrease with a decrease in their concentrations in the donor. Other mechanisms may be involved in the appearance of the steady-state transport of diclofenac in the presence of either antioxidant. For example, different concentrations of the antioxidant may have different effects on protein conformation and on permeability.

The reduction in the lag time which accompanies an increase in the concentration of these antioxidants may be due to rapid hydration of the skin surface by an increase in protein thiol. Since diclofenac is present in the ionized form in the medium and it is likely that the transport of the ionized form occurs via an aqueous route, rapid hydration may result in a reduction in the lag time for diclofenac transport.

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