Cutaneous Metabolism of Nitroglycerin *in Vitro*. II. Effects of Skin Condition and Penetration Enhancement

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The effects of skin storage, skin preparation, skin pretreatment with a penetration enhancer, and skin barrier removal by adhesive tape-stripping on the concurrent cutaneous transport and metabolism of nitroglycerin (GTN) have been studied *in vitro* using hairless mouse skin. Storing the skin for 10 days at 4°C did not alter barrier function to total nitrate flux [GTN + 1,2-glyceryl dinitrate (1,2-GDN) + 1,3-glyceryl dinitrate (1,3-GDN)]. However, metabolic function was significantly impaired and suggested at least fivefold loss of enzyme activity. Heating skin to 100°C for 5 min appreciably damaged hairless mouse skin barrier function. The ability to hydrolyze GTN was still present, however, and remained constant over the 10-hr experimental period, in contrast to the "control," which showed progressively decreasing enzymatic function with time. Pretreatment of hairless mouse skin *in vivo* (prior to animal sacrifice, tissue excision, and *in vitro* transport/metabolism studies) with 1-dodecylazacycloheptan-2-one (Azone), a putative penetration enhancer, significantly lowered the skin barrier to nitrate flux (relative to the appropriate control). Again, barrier perturbation resulted in essentially constant metabolic activity over the observation period. The ratio of metabolites formed (1,2-GDN/1,3-GDN) was increased from less than unity to slightly above 1 by the Azone treatment. Adhesive tape-stripping gradually destroyed skin barrier function by removal of the stratum corneum. The effects of 15 tape-strips were identical to those of Azone pretreatment: a greatly enhanced flux, a constant percentage formation of metabolites over 10 hr (once again), and an increase in the 1,2-GDN/1,3 GDN ratio. Overall, the experiments caution that, for transdermal drug delivery candidates susceptible to skin metabolism, the status of barrier function (enhancer pretreated, skin damage or disease, etc.) may significantly affect systemic availability.

KEY WORDS: nitroglycerin; cutaneous metabolism; transport; penetration enhancer; Azone.

INTRODUCTION

In a previous paper (1), we investigated nitroglycerin (GTN) metabolism during its percutaneous transport across hairless mouse skin *in vitro*. The results were compared to the conversion of GTN to its 1,2- and 1,3-glyceryl dinitrate metabolites (1,2-GDN and 1,3-GDN, respectively) by the 9000*g* supernatant of homogenized skin. It was found that the results from the homogenate did not predict the metabolism profile occurring during GTN transport across intact skin and, further, could not differentiate between the different levels of metabolism observed following GTN delivery to the skin surface in different topical formulations.

In this paper, we extend these, and other, earlier investigations (2) to address additional issues pertinent to the transdermal delivery/metabolism question. We again employ the transport of GTN across, and its conversion to GDN metabolites by, hairless mouse skin *in vitro* as the model system for study. Specifically, we have considered the effects on GTN transport and metabolism of (a) storing the skin at lowered temperatures, (b) briefly heating the skin to 100°C, (c) pretreating the skin with a putative penetration enhancer (1-dodecylazacycloheptan-2-one; Azone) (3), and (d) progressively removing the stratum corneum (skin's outermost and least permeable layer) by adhesive tape-stripping.

EXPERIMENTAL

Materials

Nitroglycerin solution (Tridil, American Critical Care, Agunadilla, PR) was diluted with pH 7.4 phosphate-buffered saline (PBS) to a final concentration of 0.1 mg/ml. Azone (1-dodecylazacycloheptan-2-one) was a gift from Whitby Pharmaceuticals (Richmond, VA) and was used as received. All other chemicals were reagent grade or better (Sigma Chemical Co., St. Louis, MO) and were used without further purification. Hairless mice (SKH:HR-1), aged 10–15 weeks, were purchased from the Skin Cancer Hospital, Philadelphia, PA. Full-thickness, dorsal skin was removed postsacrifice and was then either used immediately or stored as described below.

Nitroglycerin (GTN) and Glyceryl Dinitrate (GDN) Assay

The procedures and apparatus used were identical to those described previously (1).

Skin Pretreatments

The transport and metabolism of GTN were evaluated following administration of the aqueous drug solution to hairless mouse skin pretreated as follows.

(i) *Control:* After sacrifice of the animal, the skin was removed and used immediately.

(ii) *Stored:* Immediately postmortem, the skin was excised, wrapped tightly in aluminum foil, and stored at 4°C for 10 days.

(iii) *Heated:* The excised skin was placed in a plastic bag, which was then immersed in boiling water for 5 min. After cooling, the skin was used immediately.

(iv) *Azone-treated:* The enhancer was dissolved in propylene glycol to give a 2% (w/w) solution, 0.3 ml of which was used to saturate the absorbent pad of a polypropylene chamber (Hilltop Research, Inc., Cincinnati, OH). The chamber was then applied to the back of a hairless mouse *in vivo* for 14 hr. The chamber was taped securely to the animal. At the end of

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the application period, the chamber was removed and the skin wiped clean. The animal was then sacrificed and the skin removed as described before for immediate study. Control experiments, in which (a) propylene glycol alone was applied or (b) simply the chamber without any additives was used, were also performed.

(v) *Stripped:* Following sacrifice of the mouse, the skin was excised and then tape-stripped, to remove the stratum corneum, either 5, 10, or 15 times. Stripping was accomplished using Scotch Magic Transparent Tape (3M Co., St. Paul, MN).

Percutaneous Penetration

Skin (0.95 cm^2), which had been subjected to one of the above pretreatments, was mounted in a flow-through diffusion cell (Laboratory Glass Apparatus, Inc., Berkeley, CA) (4), thermostated at 35°C. The receptor compartment of the cell was perfused with pH 7.4 PBS at about 5 ml/hr (i.e., sufficient to exchange the contents completely in 1 hr). To initiate the permeation experiment, 1 ml of aqueous GTN solution (0.1 mg/ml) was introduced into the donor compartment. Subsequently, over the next 10 hr, samples of the receptor phase were collected hourly on a programmable fraction collector (Gilson PC220, Gilson Co., Inc., Middleton, WI). These samples were frozen and later processed and analyzed for nitrate (GTN and GDNs) content. All skin permeation measurements were performed in at least quadruplicate.

RESULTS AND DISCUSSION

The principal goals of our experiments examining the cutaneous metabolism of GTN are (a) to assess the extent to which GTN is subject to a dermal "first-pass effect" when topically administered and (b) to evaluate the usefulness of *in vitro* measurements for the determination of skin metabolism. In this paper, we have attempted to examine the impact of several variables (skin storage, pretreatment, the effect of penetration enhancement, and skin damage) on the biotransformation of GTN by skin.

Figures 1 and 2 report the effect of skin storage. It is not uncommon in percutaneous penetration experiments to use skin which has been excised (from an animal or human cadaver) and then stored for various periods at reduced temperature. While it has been shown that such storage may cause marginal perturbation of barrier function, it is generally expected that metabolic activity of the skin will be destroyed (5). Figures 1 and 2 are consistent with these expectations. Figure 1 shows that total nitrate flux across hairless mouse skin is not significantly ($P > 0.05$) altered by storing the skin for 10 days in the refrigerator. Figure 2, on the other hand, demonstrates that storage severely compromises skin metabolic activity. We note, however, that a small residual ability to reduce GTN remained.

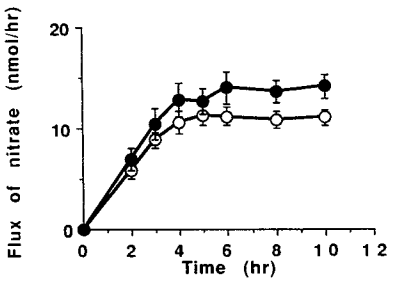

Fig. 1. *In vitro* flux (mean ± SE) of total nitrate species (GTN + 1,2-GDN + 1,3-GDN) across hairless mouse skin which was removed at sacrifice and used immediately (n = 8) (open circles) and which was removed, then stored for 10 days at 4°C before use (n = 6) (filled circles).

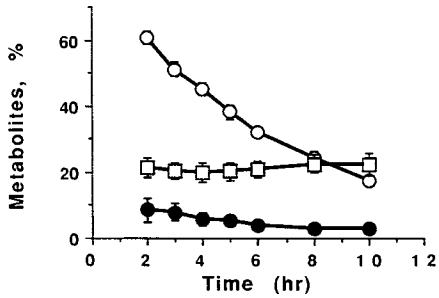

Fig. 2. Percentage (mean ± SE) of metabolites (1,2-GDN + 1,3-GDN) penetrating to the receptor phase, as a function of time, following application of GTN to hairless mouse skin which was removed at sacrifice and used immediately (n = 8) (open circles), which was removed, then stored for 10 days at 4°C before use (n = 6) (filled circles), and which was removed, then heated at 100°C for 5 min before use (n = 5) (open squares).

Although we have used full-thickness skin in the experiments reported here, percutaneous absorption *in vitro* is often measured (particularly with human skin) using separated epidermis (6). A common technique to isolate epidermis from dermis is to heat the tissue briefly and then to separate the two layers carefully by peeling one from the other with forceps. We have therefore investigated the effect of heating the skin membrane on metabolic function (although it must be said that our heating procedure was more aggressive than that typically used to separate epidermis from dermis). Table I reports the fluxes of GTN, 1,2-GDN and 1,3-GDN across control and heated skins. Figure 2 includes some of these data in graphical form. The following observations are apparent.

(1) The total nitrate flux across the heated skin was significantly greater ($P < 0.01$) than that through the control membrane. Clear alteration of the barrier function of the stratum corneum is suggested, therefore.

(2) The metabolic ability of the heated skin to convert GTN to GDNs was measurable and was constant throughout the 10-hr experimental period. Because of the greater nitrate flux through the heated membrane, GDNs were actually formed in higher amounts (relative to the control) by the heated tissue. The heating procedure used, therefore, did not completely destroy the activity of the skin. A residual capability to (nonenzymatically?) reduce GTN remained.

(3) However, the biotransformation behavior, compared to the control skin, is different. Table 1, for example, shows a significant difference in the 1,2-

Table I. Nitrate Transport (Mean ± SD) into the Receptor Phase as a Function of Time Following Application of GTN in PBS to Untreated and to Heat-Treated Hairless Mouse Skin *in Vitro*

Time (hr)	Nanomoles per hour GTN	1,2-GDN	1,3-GDN	Ratio[a]
Control				
2	1.9 ± 0.8	1.6 ± 0.6	2.0 ± 0.8	0.8 ± 0.06
3	3.7 ± 1.4	2.1 ± 0.4	2.5 ± 1.0	0.9 ± 0.08
4	5.0 ± 2.0	1.3 ± 0.6	2.5 ± 1.0	0.9 ± 0.08
5	6.0 ± 2.6	2.0 ± 0.4	2.3 ± 0.8	0.9 ± 0.06
6	6.5 ± 2.6	1.7 ± 0.4	1.9 ± 0.6	0.9 ± 0.08
8	7.0 ± 2.8	1.3 ± 0.4	1.3 ± 0.4	1.0 ± 0.08
10	7.8 ± 3.0	1.0 ± 0.2	1.0 ± 0.4	1.0 ± 0.08
Heated skin				
2	11.7 ± 6.6	1.5 ± 0.4	2.1 ± 1.0	0.7 ± 0.04
3	16.8 ± 9.4	2.1 ± 0.8	2.9 ± 1.4	0.7 ± 0.04
4	20.3 ± 11.0	2.4 ± 0.8	3.4 ± 1.4	0.7 ± 0.06
5	18.1 ± 9.2	2.3 ± 0.6	3.1 ± 1.2	0.7 ± 0.06
6	17.3 ± 8.4	2.3 ± 0.6	3.1 ± 1.2	0.7 ± 0.06
8	14.5 ± 7.0	2.2 ± 0.8	3.0 ± 1.4	0.7 ± 0.06
10	12.4 ± 5.8	1.8 ± 0.6	2.4 ± 1.2	0.8 ± 0.06

[a] Ratio 1,2-GDN/1,3-GDN.

GDN/1,3-GDN ratio of metabolites formed by control and heated skins. Heating the skin leads to preferential formation of 1,3-GDN over 1,2-GDN at all measurement times (ratios significantly different at $P < 0.05$).

Pretreatment of hairless mouse skin with a 2% Azone solution in propylene glycol significantly enhanced ($P < 0.01$) the total flux of nitrate species (Fig. 3) compared to the three controls [(a) no pretreatment, (b) pretreatment with occlusion, and (c) pretreatment with propylene glycol containing no enhancer]. As observed previously, this enhanced transport was accompanied by a relatively lower level of metabolite formation (Fig. 4). In contrast to our previous study (1), where we found that formulation changes (replacing a solution with an ointment or with a transdermal delivery system), which enhanced nitrate flux, did not alter the 1,2-GDN/1,3-GDN ratio, the incorporation of propylene glycol (and/or Azone) into the applied vehicle caused elevated formation ($P < 0.05$) of 1,2-GDN compared to the 1,3-isomer [ratios of 1,2-GDN/1,3-GDN were as follows: control, 0.8 ± 0.05; propylene glycol (PG), 1.0 ± 0.08; Azone/PG, 1.1 ± 0.15; the control value is significantly lower than the other two]. The structural similarity between the nitrates and propylene glycol may account for this observation, although precise mechanistic conclusions cannot be deduced from the data available. The possibility of an enzyme inhibitory effect of Azone itself cannot be excluded, given that the greatest impact on the 1,2-GDN/1,3-GDN ratio takes place following pretreatment with the enhancer. Note, in this regard, the similarity between the Azone profile in Fig. 4 and the heated skin profile in Fig. 2. It must be emphasized, however, that, during the 10-hr period of the experiment, the skin is losing a substantial fraction of its metabolizing capability. While the prolonged duration of the study allows us to view the full details of the nitrate permeation profiles, the metabolism information in the 2-hr data is probably most relevant to the *in vivo* situation, since it is within this period that the skin remains in reasonably good biochemical shape.

Finally, the effect of tape-stripping the skin prior to GTN application is addressed in Figs. 5 and 6. Tape-stripping is frequently employed in skin permeation research as a tool by which the effect of skin damage or disease, on the availability of topically applied chemicals, may be assessed. In a fashion similar to that described by other labo-

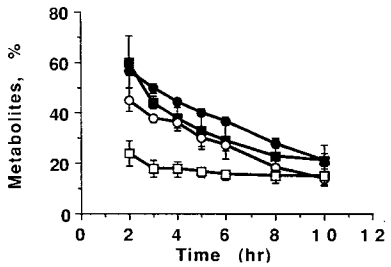

Fig. 4. Percentage (mean ± SE) of metabolites (1,2-GDN + 1,3-GDN) penetrating to the receptor phase, as a function of time, following application of GTN to hairless mouse skin, subjected to the pretreatments described in the legend to Fig. 3.

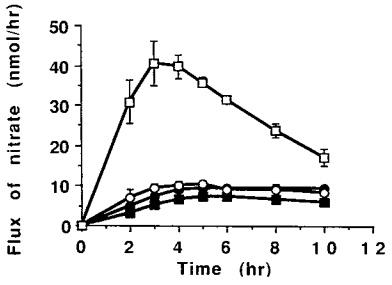

Fig. 3. *In vitro* flux (mean ± SE) of total nitrate species (GTN + 1,2-GDN + 1,3-GDN) across hairless mouse skin which was removed at sacrifice and used immediately ($n = 4$) (filled circles), which was occluded for 14 hr *in vivo*, using a polypropylene chamber, then removed at sacrifice and used immediately ($n = 3$) (filled squares), which was treated with propylene glycol (PG) under occlusion for 14 hr *in vivo*, then removed at sacrifice and used immediately ($n = 4$) (open circles), and which was treated with a 2% (v/v) solution of Azone in PG under occlusion for 14 hr *in vivo*, then removed at sacrifice and used immediately ($n = 5$) (open squares).

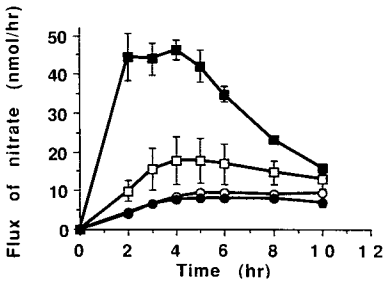

Fig. 5. *In vitro* flux (mean ± SE) of total nitrate species (GTN + 1,2-GDN + 1,3-GDN) across hairless mouse skin which was removed at sacrifice and used immediately ($n = 4$) (open circles), stripped 5 times with adhesive tape and then used immediately ($n = 3$) (filled circles), tape-stripped 10 times and then used immediately ($n = 4$) (open squares), and tape-stripped 15 times and then used immediately ($n = 5$) (filled squares).

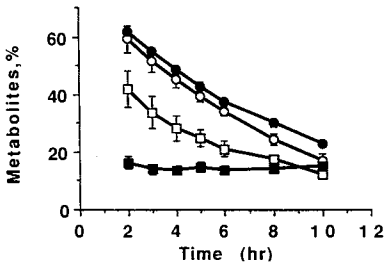

Fig. 6. Percentage (mean ± SE) of metabolites (1,2-GDN + 1,3-GDN) penetrating to the receptor phase, as a function of time, following application of GTN to hairless mouse skin subjected to the pretreatments described in the legend to Fig. 5.

ratories using different permeants (7), tape-stripping the stratum corneum progressively destroys barrier function. A comparison of Figs. 3 and 5 shows that pretreatment of hairless mouse skin with 2% (v/v) Azone is essentially equivalent to complete removal of the stratum corneum, confirming the previously established sensitivity of the murine skin to Azone treatment (8). The impact of tape-stripping on GDN metabolite formation is illustrated in Fig. 6. The similarity between the Azone pretreatment (see Fig. 4) and the 15-tape-strip experiment is again striking. Further, the cumulative permeation of GTN and the GDN metabolites is very consistent between the Azone pretreatment and the 15-tape-strip studies. Additionally, the change in the 1,2-GDN/1,3-GDN ratio is essentially identical to the enhancer-treated situation [ratio of 1,2-GDN/1,3-GDN following 15 tape-strips was 1.1 ± 0.15; the corresponding control value (0.8 ± 0.05) was significantly ($P < 0.05$) less]. As the tape-stripping experiment involves only a physical perturbation of the skin, this finding argues against an enzyme-inhibition action for the enhancer. When the skin has been most severely compromised (15 tape-strips), the enzyme activity (measured by percentage GDN metabolite formation; Fig. 6) is essentially constant over the entire 10-hr period of the experiment (as observed for the Azone profile in Fig. 4 and the "heated skin" profile in Fig. 2). The precise explanation for the results obtained cannot be unequivocally deduced.

In summary, the results presented in this paper serve as important reminders of the complexity of the percutaneous transport/metabolism problem. While the experiments have been conducted using hairless mouse skin, an animal model that typically amplifies the behavior to be expected in human skin (9,10), the observations are nevertheless pertinent because they illustrate the wide range of phenomena which may occur. The data certainly confirm that any attempt to generate meaningful metabolism information from stored or heat-treated skin is likely to be unsuccessful; they show that the metabolic function of (and, hence, the quality of information from) freshly excised, "normal," control skin deteriorates with time postremoval from the animal; and they indicate that lowering the barrier function of skin can significantly change the metabolic outcome. It follows that transdermal drug delivery candidates, which are believed to be susceptible to dermal metabolism, must be carefully evaluated, particularly if coadministration with a permeation enhancer is contemplated.

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