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Effect of 2,4-Dinitrophenol on Transport of Cefmetazole and Diclofenac from Thigh Muscle Tissue and from Intestinal Connective Tissue into Blood Circulation in Rats

TOSHIAKI NISHIHATA,*,[a] TAKUMI SUZUKA,[b] NOBORU YATA,[b] and KIYOSHI SAKAI[c]

Pharmaceutical Chemistry Department, University of Kansas,[a] 2065 Constant Avenue, Lawrence, Kansas 66046, U.S.A., Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine,[b] 1–2–3 Kasumi, Minamiku, Hiroshima 734, Japan, and Osaka University of Pharmaceutical Sciences,[c] 2–10–65 Kawai, Matsubara, Osaka 580, Japan

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2,4-Dinitrophenol (DNP), which caused rapid protein thiol (PSH) loss in tissue of muscle and intestine, resulted in decreased drug transport from muscle tissue and from intestinal connective tissue into the blood circulation. The effect of DNP in decreasing the transport of cefmetazole was greater than that in decreasing the transport of diclofenac, suggesting that PSH loss in the tissue may decrease the apparent diffusion of solute in the tissue fluid through the aqueous pathway. Trifluoperazine inhibited the rapid PSH loss caused by DNP with a recovery of drug transport; this also supports the view that PSH loss may be involved in the mechanism of decrease in the diffusion of solute by DNP.

Keywords——transport; muscle; intestine; cefmetazole; diclofenac; rat; 2,4-dinitrophenol; trifluoperazine; thiol

Glutathione, a major nonprotein thiol (NPSH), and protein thiol (PSH) play an important role in maintenance of integrity of tissue and cells. Depletion of NPSH can induce gastric ulcer[1] and hepatic disorder.[2] The importance of both PSH and NPSH in maintenance of intracellular Ca^{2+} homeostasis has been established in studies using isolated rat hepatocytes.[3,4]

Regarding the role of the thiols in the transport of organic compounds, there is a report[5] showing that gluthathione mediates intestinal absorption of L-amino acid. We have recently reported that NPSH loss in rat intestinal epithelium caused an increase in passive transport of phenol red and cefmetazole, hydrophilic compounds, across the intestinal epithelium.[6–8] However, we have also reported that PSH loss induced by a high concentration of diethyl maleate decreased the transport of cefmetazole across the intestinal epithelum and also overcame the effect of NPSH loss in increasing cefmetazole transport.[8] Except for these findings, essentially nothing is known about the influence of thiols on drug transport across the tissue. For example, transport of a drug into the blood circulation after injection into muscle tissue may be a critical factor in the appearance of the therapeutic effect of the drug.

It is known that 2,4-dinitrophenol (DNP) acts as an uncoupler to deplete reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) in mitochondria and that the depletion of NAD(P)H leads to failure to reduce disulfides to thiols.[9] In the present study, we investigated the effect of DNP on the thiol levels in muscle tissue and in intestinal connective tissue of rats, as well as the effect of DNP on drug transport into the blood circulation after injection of a drug into thigh muscle and into intestinal connective tissue of rats.

Experimental

Materials——DNP and bradykinin were obtained from Sigma Chemicals Co. (St. Louis, U.S.A.). Trifluoperazine malonate (TFPZ) was supplied by Yoshitomi Pharmaceutical Industry (Osaka, Japan). Sodium cefmetazole and sodium diclofenac were supplied by Sankyo Co., Ltd. (Tokyo, Japan) and Japan Ciba Geigy (Takarazuka, Japan), respectively. Other reagents used were of analytical grade.

Rat Experiments——Male Wistar rats, 250 to 300 g, were fasted for 16 h prior to experiments. During the experiment rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.) and were kept on a warm surface (electric hot plate) at 38 °C to maintain body temperature.

Drug transport into the blood after injection into thigh muscular tissue was examined as follows: 20 μl of drug solution (saline) was injected together with DNP into thigh muscular tissue and then blood samples were collected from the jugular vein at designated time intervals.

In terms of an investigation of the transport from intestinal connective tissue, an about 2 cm intestinal segment was ligated at the proximal end 20 cm from the bile duct after abdominal incision. A 20 μl aliquot of drug solution (saline) was injected into intestinal connective tissue in which the ligated loop had been prepared, at 30 min after administration of 200 μl of DNP solution (saline) into the lumen of the intestinal loop. After the injection, blood samples were collected. After centrifugation of blood, the plasma was used for measurement of the drug concentrations.

In a separate study, thigh muscle tissue (about 200 mg) around the injection area and the small intestinal segment of loop were removed after the administration of DNP, and homogenized in 0.1 M sodium phosphate-buffered solution (pH 7.2) after being rinsed with saline. The PSH and NPSH contents were determined.

Assay Procedures——Assays of cefmetazole[10] and diclofenac[11] in rat plasma were performed by a high-performance liquid chromatographic method as described previously. Assays of both PSH and NPAH were performed by the method of Di Monte *et al.*[3] Assay of protein in the homogenate was done by the method of Lowry *et al.*[12]

TABLE I. Effect of DNP[a] on Nonprotein Thiol and Protein Thiol in Rat Intestinal Tissue and Thigh Muscle Tissue

Treatment number and time after administration (h)	Thiol levels (μmol/g-protein)			
	Intestinal tissue		Muscle tissue	
	Nonprotein	Protein	Nonprotein	Protein
No additive	2.06±0.27	102.7±16.1	1.46±0.21	109.1±11.4
(1) Saline			(1) Saline	
0.5	2.14±0.11	98.4± 8.7	1.55±0.31	110.2±19.4
1	2.09±0.26	104.6±11.7	1.42±0.17	102.6±17.2
3	2.12±0.16	100.2±11.4	1.51±0.06	104.7± 8.6
5	2.28±0.29	106.4±18.6	1.47±0.08	107.4±11.1
(2) DNP at 1 nmol			(2) DNP at 0.1 nmol	
0.5	2.24±0.40	103.7±14.1	1.36±0.14	109.4±11.4
1	1.98±0.32	94.4± 8.5	1.41±0.21	102.4±12.3
3	2.01±0.36	94.6± 7.8	1.31±0.08	93.5±14.2
5	2.23±0.41	99.7± 5.6	1.39±0.23	102.4±12.6
(3) DNP at 20 nmol			(3) DNP at 2 nmol	
0.5	2.08±0.26	86.4±12.2	1.40±0.09	79.8± 6.7[b]
1	2.02±0.09	72.9± 5.6[b]	1.26±0.14	72.6± 8.1[b]
3	1.64±0.20	80.1± 9.1	0.89±0.09[b]	75.1±10.8[b]
5	1.98±0.44	96.4±11.6	1.29±0.26	90.2±10.4
(4) DNP at 200 nmol				
0.5	2.03±0.26	72.3±10.7[b]		
1	1.84±0.31	64.4± 5.6[b]		
3	1.41±0.28[b]	76.2± 8.9[b]		
5	1.76±0.16[b]	86.6±11.7		

a) Administration of DNP in a volume of 200 μl into an intestinal loop or 20 μl into thigh muscle was carried out as described in the experimental section. Each value represents the mean±S.D. ($n=3$ for saline and $n=4$ to 7 for others). *b*) $p<0.05$ *versus* (1).

TABLE II. Effect of TFPZ and Bradykinin on Nonprotein Thiol and Protein Thiol in Rat Intestinal Tissue and Thigh Muscle Tissue

Treatment number and time after administration (h)	Thiol levels (μmol/g-protein)			
	Intestinal tissue		Muscle tissue	
	Nonprotein	Protein	Nonprotein	Protein
(5) TFPZ[a] at 3 nmol			(5) TFPZ[b] at 1 nmol	
1	2.12±0.14	99.7±11.6	1.49±0.14	101.6±12.7
3	2.11±0.16	104.2±14.3	1.54±0.19	107.1±16.5
(6) DNP at 200 nmol+ TFPZ[a] at 3 nmol			(7) DNP at 20 nmol+ TFPZ[b] at 1 nmol	
0.5	1.79±0.16	99.2± 9.1[d]	1.41±0.08	101.7± 9.2[d]
1	1.59±0.10[c]	88.7±12.6[d]	1.17±0.11	86.5±10.6[d]
3	1.61±0.19[c]	85.2±10.8	0.92±0.07[c]	85.2± 8.1
5	1.81±0.27	94.1±19.2	1.31±0.20	99.6±12.7
(8) Bradykinin[a] at 3 nmol			(8) Bradykinin[b] at 1 nmol	
1	2.16±0.21	117.4±20.3	1.51±0.16	110.2±11.6
3	2.09±0.09	101.6±11.4	1.49±0.08	109.4± 9.7
(9) DNP at 200 nmol+ bradykinin[a] at 3 nmol			(10) DNP at 20 nmol+ bradykinin[b] at 1 nmol	
0.5	2.04±0.19	70.6± 9.4[c]	1.44±0.19	77.6±13.4[c]
1	1.81±0.21	65.7± 7.2[c]	1.32±0.24	64.2± 8.7[c]
3	1.57±0.14[c]	74.2± 9.4[c]	0.75±0.08[c]	73.6± 8.2[c]
5	1.87±0.19	91.6±17.6	1.31±0.14	91.4± 8.6

a) TFPZ or bradykinin was injected into the intestinal connective tissue (1 nmol at each of three places) at 30 min after the administration of DNP into the intestinal lumen. *b*) TFPZ or bradykinin was injected together with DNP. Each value represents the mean±S.D. ($n=4$ to 7). *c*) $p<0.05$ *versus* (1) in Table I. *d*) For (6) and (7), $p<0.05$ *versus* (4) and (3) in Table I, respectively.

Statistical analysis were performed by means of Student's *t*-test.

Results

The effect of DNP on both PSH and NPSH in the thigh muscle tissue was examined. An injection of DNP at 2 nmol caused rapid PSH loss but only a slow decrease in NPSH (Table I). Similar results were observed in the ligated intestinal tissue, when DNP (20 or 200 nmol) was administered in the ligated intestinal lumen. When DNP was administered at 2 nmol into the muscle or at 20 nmol into the intestinal lumen, both PSH and NPSH in thigh muscle and intestine recovered after 5 h (Table I). When DNP (6 nmol) was injected into intestinal connective tissue of the loop (20 μl of solution was injected at each of three places), rapid PSH loss was observed with 76.9±7.6 μmol/g-protein after 0.5 h ($n=3$, $p<0.05$ *versus* (1) in Table I) and 70.4±4.9 μmol/g-protein after 1 h ($n=3$, $p<0.05$ *versus* (1) in Table I). These results may indicate that intestinal absorption of DNP is slower than expected and/or dilution of DNP occurs in the tissue.

Coadministration of TFPZ at 1 nmol with DNP inhibited the loss of PSH, especially at the early stage (at 0.5 and 1 h) in the thigh muscle, though there was no inhibition of NPSH loss (Table II). An injection of TFPZ at 1 nmol in the connective tissue of the ligated intestinal loop at 30 min after the administration of DNP in the lumen also seemed to restore only PSH in the tissue at early stage (at 0.5 and 1 h; see Tables I and II). An administration of bradykinin did not affect the levels of these thiols (Table II).

The appearance of cefmetazole and diclofenac in plasma after injection into connective

TABLE III. Appearance of Cefmetazole and Diclofenac in Plasma after Injection[a] of Cefmetazole (1 mg in a Rat) and Sodium Diclofenac (0.25 mg) with TFPZ (1 nmol) or Bradykinin (1 nmol) into Connective Tissue of the Ligated Small Intestine 30 min after Administration of DNP[b] into the Lumen of an Intestinal Loop

Treatment No. Additive (nmol)	Plasma drug concentration (μg/ml)		
	15	30	60 (min)
Cefmetazolo			
(1) None	3.52±0.53	3.17±0.42	2.43±0.36
(2) DNP (20)	2.14±0.42[c]	1.87±0.51[c]	1.79±0.56
(3) DNP (200)	1.59±0.33[c]	1.53±0.42[c]	1.46±0.41[c]
(4) DNP (200)+TFPZ	3.02±0.21[d]	3.07±0.32[d]	2.47±0.41
(5) TFPZ	3.60±0.42	3.19±0.27	2.54±0.21
(6) DNP (200)+bradykinin	2.02±0.46[c]	2.26±0.17[c]	1.97±0.14[c]
(7) Bradykinin	6.62±0.71[c]	6.04±0.96[c]	4.17±0.32[c]
(8) Administration of cefmetazole 5 h after DNP (20)	2.79±0.26	2.62±0.26	2.49±0.27
Diclofenac			
(1) None	4.24±0.52	4.03±0.46	1.96±0.54
(2) DNP (20)	3.96±0.41	3.74±0.56	1.99±0.46
(3) DNP (200)	3.29±0.36	3.16±0.47	2.12±0.37
(4) DNP (200)+TFPZ	4.04±0.36	4.16±0.32	1.84±0.49
(5) TFPZ	4.39±0.21	3.98±0.23	1.87±0.42
(6) DNP (200)+bradykinin	3.42±0.36	3.24±0.29	2.36±0.42
(7) Bradykinin	4.96±0.42	4.17±0.31	2.14±0.62
(8) Administration of diclofenac 5 h after DNP (20)	3.91±0.31	3.87±0.42	2.02±0.16

a) 20 μl of solution was injected. *b*) 200 μl of solution was administered. Each value represents the mean±S.D. ($n=4$ to 5). *c*) $p<0.05$ *versus* (1). *d*) $p<0.05$ *versus* (3).

tissue of the ligated small intestinal loop was delayed by the preadministration of DNP into the ligated intestinal lumen (Table III). The delay of cefmetazole appearance in plasma by DNP treatment was more marked than that of diclofenac. The simultaneous injection of DNP with both drugs into thigh muscle tissue also delayed the appearance of drugs in plasma. A significant delay of cefmetazole appearance was also observed in comparison with diclofenac (Table IV). The appearance of both drugs following administration at 5 h after the administration of DNP showed similar concentration profiles in plasma to those without administration of DNP (Tables III and IV).

The appearance of both drugs in plasma after injection into thigh muscle tissue, which was delayed by the administration of DNP, was restored by the coadministration of TFPZ with the drugs (Table IV). The administration of TFPZ with the drugs into intestinal connective tissue at 30 min after the administration of DNP also restored the appearance of both drugs in plasma (Table III).

Coadministration of bradykinin with the drugs into thigh muscle tissue or into intestinal connective tissue accelerated the appearance of cefmetazole in plasma. However, bradykinin did not restore the appearance of drugs which was delayed by DNP administration (Tables III and IV).

The administration of DNP into the intestinal lumen or into muscle tissue did not influence the concentration of diclofenac or cefmetazole in plasma during 1 h after intravenous administration of both drugs (data not shown).

TABLE IV. Appearance of Cefmetazole and Diclofenac in Plasma after Injection[a] of Cefmetazole (1 mg in a Rat) and Diclofenac (0.25 mg) into Muscle with DNP and TFPZ (1 nmol) or Bradykinin (1 nmol)

Treatment No. Additive (nmol)	Plasma drug concentration (μg/ml)		
	15	30	60 (min)
Cefmetazole			
(1) None	2.41±0.43	2.92±0.37	2.68±0.21
(2) DNP (2)	1.08±0.24[b]	1.34±0.16[b]	1.69±0.27[b]
(3) DNP (2)+TFPZ	1.98±0.42	2.71±0.38[b]	2.73±0.28[c]
(4) TFPZ	2.37±0.29	2.89±0.36	2.71±0.18
(5) DNP (2)+bradykinin	1.64±0.11[b]	1.82±0.27[b]	2.54±0.34
(6) Bradykinin	4.92±0.31[b]	4.56±0.26[b]	3.41±0.32[b]
(7) Administration of cefmetazole 5 h after NDP (2)	2.01±0.24[c]	2.47±0.46[c]	2.64±0.32[c]
Diclofenac			
(1) None	3.34±0.31	3.46±0.36	2.71±0.33
(2) DNP (2)	2.58±0.29	2.43±0.32[b]	2.37±0.46
(3) DNP (2)+TFPZ	3.16±0.36	3.52±0.41[c]	2.87±0.42
(4) TFPZ	3.52±0.40	3.54±0.37	2.82±0.41
(5) DNP (2)+bradykinin	2.74±0.16	2.89±0.14	2.51±0.42
(6) Bradykinin	3.94±0.06	3.51±0.42	2.67±0.26
(7) Administration of diclofenac 5 h after DNP (2)	3.16±0.29	3.27±0.42	3.01±0.36

a) 20 μl of solution was injected. Each value represents the mean±S.D. ($n=4$ to 5). *b*) $p<0.05$ *versus* (1). *c*) $p<0.05$ *versus* (2).

Discussion

DNP caused rapid PSH loss in intestinal tissue as well as in thigh muscle tissue. Although it is considered that oxidation of NAD(P)H by the administration of an uncoupler such as DNP induces the loss of both thiols,[9] NPSH loss was slow in comparison to PSH loss in the present study (Table I). Further, TFPZ, a calmodulin inhibitor at a dose of 1 nmol,[13] inhibited only the PSH loss at the early stage after administration of DNP (Table I). It has been reported[8] that TFPZ inhibited PSH loss caused by a high concentration of diethyl maleate, even though there was no inhibition of NPSH loss. It has been also reported[14] that NAD(P)H loss in mitochondria caused by the uncoupler induced a rapid increase in cytosolic Ca^{2+} by depletion of the mitochondrial Ca^{2+} pool. From these findings, it is supposed that rapid PSH loss induced by DNP occurs by a mechanism probably involving intracellular Ca^{2+}, rather than the oxidation mechanism which is also involved in the losses of both PSH and NPSH induced by DNP.

The effect of DNP in suppressing transport of cefmetazole and diclofenac from thigh muscle tissue or from intestinal connective tissue to plasma seems to be related to the effect of DNP in causing PSH loss, since TFPZ inhibited both effects of DNP (Tables II, III and IV). The onset of delay of drug appearance in plasma by the administration of DNP was rapid, as was PSH loss, but NPSH loss was observed after PSH loss (Table I). Thus, PSH loss rather than NPSH loss seems to be related to the slow transport of both drugs, suggesting that disulfide bridge formation of tissue protein with PSH loss reduces the diffusitivity of the solute *via* the aqueous pathway in tissue.

The significant decrease of plasma cefmetazole concentration after injection into intestinal connective tissue or into the muscle tissue in comparison with that of diclofenac in

the presence of DNP, supports the idea that the decrease in drug transport by PSH loss in tissues is due to the decrease in the apparent diffusion of solute in the tissue fluid through the aqueous pathway, but PSH loss in tissues may not decrease the transport through the lipoidal layer. That is because cefmetazole is a hydrophilic drug and diclofenac is a lipophilic drug.

It might be considered that the effect of DNP in causing slow appearance of cefmetazole in the blood circulation is due to a reduction in blood flow rate by DNP as an adenosine triphosphate inhibitor. However, since the change in diclofenac appearance in the blood was slight, the decrease in diffusion *via* the aqueous pathway in the tissue rather than a possible decrease in blood flow seems to account for the delay in the appearance of cefmetazole.

In the present study, bradykinin, which increases the permeability of the blood vessel wall,[15] did not restore the transport of drugs from the injection site into plasma, which was delayed by DNP; *i.e.*, this result may support the view that slow transport of drugs induced by DNP is due to the decrease in the diffusion of solute through the tissue along with PSH loss in the tissue. Since the effects of DNP on thiol levels in the tissue were investigated separately from the drug transport study, however, a quantitative discussion is not appropriate here. For a quantitative evaluation, a method to control the thiol levels in the tissue is required.

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