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DT Diaphorase: Increased Enzyme Activity and mRNA Expression in Oxidant Stress of Skin[1]

RILEY S. REES, M.D., GILSON J. KINGMAN, M.D.,* BELINDA CASHMER, M.S., ROBERT R. GILMONT, PH.D.,† CHRISTOPHER REEVES, B.S., MICHAEL J. WELSH, PH.D.,† AND DAVID J. SMITH, JR., M.D.

*Department of Surgery, Section of Plastic and Reconstructive Surgery, and †Department of Anatomy & Cell Biology, University of Michigan, 1500 East Medical Center Drive, Room 2130, Taubman Center, Ann Arbor, Michigan 48109-0340; and *McAuley Health Sciences Center, Ann Arbor, Michigan 48103*

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DT diaphorase is a flavoprotein that enzymaticly transfers two electrons from quinones as intermediate substrates and has been reported to increase its activity in the liver after exposure to toxicants. In this series of experiments, we tested the hypothesis that DT diaphorase also increases its activity after exposure to oxidants following gradient ischemia in skin. Using dorsal rat flaps, oxidant stress was induced immediately or during a 7-day period of preconditioning as a bipedicle flap before the distal attachment was divided. DT diaphorase activity (ΔAbs/min/100 g) or expression of message was measured during the period of preconditioning to determine the relationship between skin survival, enzyme activity, and expression of message. There was 4.7 ± 0.8 cm of skin necrosis in the distal end of acute flaps while the preconditioned flaps had no skin necrosis after the distal attachment was divided. In the acute flaps, the DT diaphorase activity was equal throughout the flap for the first 6 hr. After 24 hr of ischemia, the DT diaphorase activity was significantly higher in the proximal end of the flap (1.83 ± 0.21 ΔAbs/min/100 g) than that in the distal end (0.005 ± 0.01 ΔAbs/min/100 g), which was significant ($P < 0.05$). In the preconditioned flaps, enzyme activity did not increase but there was as 50-fold increase in DT diaphorase activity at the distal end 24 hr after they were divided ($P < 0.05$). Maximal enzyme induction of DT diaphorase activity occurred after 4 days of preconditioning and correlated with the maximal expression of mRNA. These studies provide the first evidence that DT diaphorase enzyme activity is inducible after oxidant stress. The data also suggests that DT activity remains elevated for at least 6 hr of ischemia and may be a potential source of anti-oxidant activity in ischemic skin.

INTRODUCTION

Skin necrosis following ablative head and neck surgery has disastrous consequences since it may prevent initiation of adjuvant therapy or reduce quality of life. However, little is known about the factors that cause skin flap failure. Many studies confirm the importance of free radical production in the skin as a component of tissue injury [1–3]. Our previous work [4, 5] suggests that increased oxidant production and neutrophil infiltration are associated with increased skin necrosis. These effects are reduced if the skin is subjected to ischemic preconditioning, a process plastic surgeons have termed "delay."

Since the preconditioning effect (delay) is an effective method of enhancing skin flap survival, it has been studied extensively despite its limited clinical application. McFarlene *et al.* [6] showed that dorsal rat flaps developed reproducible skin necrosis in the distal end (Fig. 1A). In his model, preconditioning of the skin as a bipedicle skin flap prevented skin necrosis when the distal attachment was divided (Fig. 1B). Extensive studies using this animal model suggest that the preconditioning results in enhanced blood flow [7], reduced neutrophil recruitment [4, 5], and enhanced enzyme activity in the glycolytic pathway [8].

In this series of experiments, we explored the enzymatic activity and molecular message expression of the flavoprotein, DT diaphorase, after preconditioning. These flavoproteins, which are diverse in character and constitutively present in our diet, may be potent antioxidants to accept electron pairs formed during free radical production. Since there is reduced oxidant activity following preconditioning of skin [4, 5], we propose that antioxidant enzyme, DT diaphorase, may play a role in reducing skin injury during ischemia.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all reagents are of the highest purity available and were purchased from the Sigma Chemical Corp. (St. Louis, MO).

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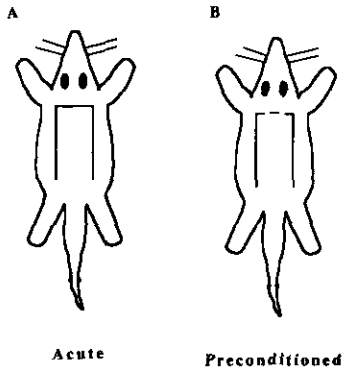

FIG. 1. (A) The rat icon depicts the 10 × 4-cm skin flap raised through the panniculous carnosis of the rat's back. The distal end near the rat's head will necrose at 7 days. The proximal end is near the tail. (B) The rat icon depicts a bipedicle skin flap on the rats back. When the distal end is divided (- - -) after 7 days, the distal end will not necrose. In some experiments, the distal end was divided at various times and enzyme activity was measured. In other experiments, the skin was assayed while the bidpedicle flap remained intact.

Animal Model

Skin flaps were raised on Sprague-Dawley rats (150–175 g) (Charles River Laboratories, Portage, MI) as shown in the rat icon (Fig. 1A). The flaps were outlined on the back with a transparent 10 × 4-cm template using the iliac crest as the base of the flap as described by McFarlene *et al.* [6]. Anesthesia was achieved with 100 mg/kg body wt ketamine hydrochloride (Parke-Davis, Morris Plains, NJ). The flaps were raised through the panniculus carnosis, immediately reapproximated with surgical staples, and excised after varying time intervals. To precondition the skin, the bipedicle skin flaps were raised through the panniculus carnosis and the distal attachment was preserved (Fig. 1B). The distal attachment was divided at various times (Fig. 1B).

Tissue specimens were collected from the skin flaps as keratome biopsies, placed on a T-shaped cutting board, and removed as 0.0012-in. skin grafts with an electric dermatome (Zimmer, Warshaw, IN). The biopsies were cooled to 4°C and tissue specimens were obtained from the proximal and distal ends of the flaps for enzyme assays and isolation of RNA.

All experiments were in accord with the standards in *The Guide for the Care and Use of Laboratory Animals* (DHEW Pub. No. (NIH) 78/23). The animal protocol was approved through the animal care committees of both the University of Michigan and the Ann Arbor Veterans Medical Center.

Experimental Groups

The animals were divided into two groups with five animals for each time period studied. In the first group, skin flaps were raised and sampled at 0, 6, 12, or 24 hr postoperatively. Skin flaps raised and harvested immediately served as sham controls for all experiments.

In the second experimental group, the bipedicle flaps were raised and secured with staples for 1, 3, 4, or 7 days. Thereafter, the flap was reelevated, the distal attachment was divided, and keratome biopies were harvested from the flaps at 0, 6, 12, and 24 hr later. These flaps were called preconditioned flaps.

DT Diaphorase Assay

The proximal and distal keratome biopies (100 mg) were weighed, suspended in cold sucrose (0.25 *M*), homogenized and sonicated, each for 60 sec, and centrifuged at 34,000 rpm for 2 hr. The supernatant cytosol was decanted and stored at −76°C until use. DT diaphorase activity was measured by the procedure described by Romyhr *et al.* [9].

The freshly prepared reaction mixture consisted of 76 μM menadione, 76 μM NADH, 76 μM NADPH, 285.7 μM nitro blue tetrazolium (NBT), and Tris buffer (pH 7.5) to a total volume of 2 ml. The reaction was started by adding 75 μl of thawed cytosol to the fresh reaction mixture. The reduction of NBT to formazan was followed spectrometrically on a Gilford Response II spectrophotometer at 530 nm. The data was expressed as Abs/min/100 mg tissue.

Assays were done in triplicate and in some experiments skin homogenates were treated with the wetting agent Tween 20 (70 ml) to enhance enzyme activity or dicumarol at a concentration of 10^{-4} *M* to abolish enzyme activity.

Studies of Flap Necrosis

In separate animal studies, to verify a reproducible model of injury, we determined the mean amount of skin necrosis ($n = 5$) at 7 days. Necrosis was defined grossly by typical signs of tissue injury including black color, eschar formation, and absence of bleeding. The total skin area of skin necrosis was traced on transparent X-ray film to determine the area. Three separate measurements for the centimeters of skin necrosis were determined and the mean value was selected for the amount of skin necrosis in the flap. The amount of skin necrosis in each group was expressed as the mean and standard error of the mean.

Isolation of Nucleic Acid

Skin samples were frozen in liquid nitrogen and maintained in a −70°C freezer until just prior to RNA isolation. RNA was isolated from tissues by a modification of the method of Chomczynski and Sacchi [10]. Briefly, tissues were homogenized in a 4 *M* guanidine thiocynate solution using a Polytron PT 3000 homogenizer (Brinkman Instruments Inc., Westbury, NY). Homogenates were centrifuged at 10,000*g* for 20 min to remove insolu-

ble material before extraction with acid phenol–chloroform. Homogenates were repeatedly extracted until no denatured protein was visable at the interface between aqueous and organic phases. Following the final precipitation from the guanidine solution, samples were washed two times with 70% ethanol and then two times precipitated from 150 m*M* NaCl solutions. Samples were lyophilized to near dryness, resuspended in water, and subjected to spectrophotometric analysis for quantitation.

Northern Blot Analysis of mRNA of DT Diaphorase

RNA samples were incubated in a buffer containing 20 m*M* Hepes (pH 7.8), 1 m*M* EDTA, 6% formaldehyde, and 50% formamide for 10 min at 65°C before electrophoresis. RNA was separated on 1.5% denaturing agarose gels and transferred onto nylon membranes (Biotrace HP; Gelman Sciences, Ann Arbor, MI). Blots were fixed by uv irradiation and prehybridized for 2 hr at 42°C in a solution containing 0.5% SDS, 400 m*M* $NaPO_4$ (pH 7.2), 1 m*M* EDTA, 1 mg/ml BSA, and 50% formamide. Radiolabeled probes were added to the mixtures and hybridizations were allowed to proceed for 18 to 24 hr. After hybridization, blots were washed in 1× SSC, 0.1% SDS two times for 30 min at 42°C. These washes were followed by three 20-min high-stringency washes in 0.2% SSC, 0.1% SDS at 65°C for cDNA probes or 55°C for oligonucleotide probes. Radiolabeled probes for DT diaphorase were prepared from a 506-bp fragment of rat NMORI 3′ cDNA inserted at the *Sma*I site of pUC13 (a gift from Daniel W. Nebert, M.D., University of Cincinnati) using [^{32}P]dCTP in a random primer reaction (Boehringer Manheim kit, Indianapolis IN). Radiolabeled probes for 18S rRNA were prepared from oligonucleotides with [^{32}P]dATP by a tailing reaction using a terminal deoxynucleotide transferase. The oligonucleotide sequence 5′-ACGGTATCTGATCGTCTTCGAACC-3′ was used to hybridize to 18S ribosomal RNA [11]. Blots were exposed to XAR-5 X-ray film (Kodak, Rochester, NY) using Kodak Lanex enhancer screens for autoradiography.

Statistical Analysis of Data

Data from each experimental group were compared and analyzed using one-way analysis of variance and Neumann–Keull post hoc testing. Data were expressed as the mean and standard error of the mean. All comparisons were made between skin obtained from the proximal and distal end of the skin flap. Statistical significance between groups were defined with a $P < 0.05$.

RESULTS

The purpose of this study was to survey DT diaphorase activity in the ischemic portion of acute and preconditioned skin flaps. Biological experiments showed no skin necrosis in preconditioned flaps and 4.7 ± 0.8 cm of skin necrosis in the distal ends of acute flaps.

The DT diaphorase activity in sham control animals was uniform throughout the length of the flap (Table 1). Enzyme activity in sham control skin was stimulated almost twofold with Tween 20, and was abolished with dicumarol. These reagents served as internal controls for tissue assays of DT diaphorase in skin homogenates.

In the acute flaps, there was gross evidence of cyanosis and decreased vascularity at 6 hr after elevation, but there was no reduction in DT diaphorase activity in the distal end of the flap. However, 24 hr later, the distal end of acute flaps had gross evidence of early necrosis and the DT diaphorase activity was reduced 50-fold in skin homogenates from the distal end (Table 1). During this period, there was a statistically significant increase in DT diaphorase activity in the proximal end of the flap compared to sham controls ($P < 0.05$). The proximal end of acute flaps was grossly viable at 24 hr and correlated with persistently increased DT diaphorase activity.

Stimulation of DT diaphorase activity in skin homogenates with Tween 20 produced a 10-fold increase in activity and suggested that DT inhibitors were present at 24 hr of ischemia. This effect was most prominant in the distal end where early skin necrosis was present. Treatment with dicumarol quenched all absorbance and suggested that other factors in the skin homogenates did not effect the assay.

TABLE 1

DT Diaphorase Activity (ΔAbs/min/100 g) Expressed in the Proximal and the Distal Ends of Skin Flaps

Hours of ischemia	Proximal			Distal		
	Dicumarol	Baseline	Tween	Dicumarol	Baseline	Tween
0	1.37 ± 0.21	2.01 ± 0.16	0.017 ± 0.01	1.83 ± 0.21	2.8 ± 0.29	0.031 ± 0.07
6	1.54 ± 0.28	2.56 ± 0.40	0.030 ± 0.01	1.66 ± 0.23	2.59 ± 0.34	0.024 ± 0.01
24	2.04 ± 0.29	3.41 ± 0.38	0.043 ± 0.01	0.04 ± 0.01*	.397 ± 0.08	0.005 ± 0.01

Note. DT diaphorase activity was stimulated with Tween 20 and abolished with dicumarol added to the skin homogenates.
* $P < 0.05$ compared to the proximal end using paired Student t tests and $N = 5$.

TABLE 2

Baseline DT Diaphorase Activity (ΔAbs/min/100 g) in Skin Flaps Preconditioned for 7 Days and Assayed at 6, 12, and 24 hr after Division of the Distal Attachment

Hours of ischemia	Proximal (baseline)	Distal (baseline)
6	2.90 ± 0.6	3.03 ± 0.27
12	3.30 ± 0.68	2.70 ± 0.36
24	2.29 ± 0.45	0.234 ± 0.03*

* $P < 0.05$ compared to the proximal end using paired Student t tests and $N = 5$.

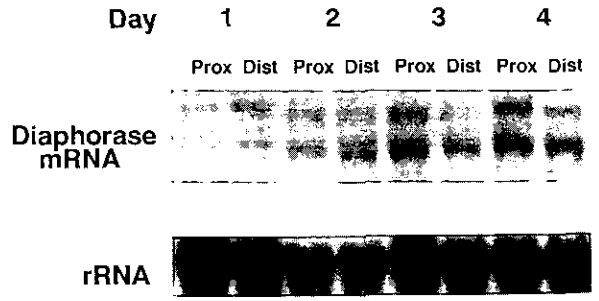

FIG. 2. Shows that the mRNA for DT diaphorase is expressed at 4 days in the proximal and distal end of the bipedicle flap. The ribosomal RNA appears constant in all lanes as a control for these experiments.

To test the hypothesis that the preconditioning of flaps enhanced DT diaphorase activity, bipedicle flaps were created for 7 days before the distal attachment was divided. The data in Table 2 show that preconditioned flaps had a twofold increase in DT diaphorase activity in both the proximal and distal ends at 6 hr ($P < 0.05$).

Both acute and preconditioned flaps experienced a decrease in DT diaphorase activity at 24 hr (Table 2). However, the distal ends of the divided bipedicle flaps appeared viable. The DT diaphorase activity in the proximal end of the flaps was maximal at 12 hr after the distal attachment was divided and was not significantly different than values in the acute flap group. The diaphorase activity was fivefold higher in the distal ends (0.23 ± 0.07) of preconditioned flaps than that in the comparable distal ends (0.04 ± 0.01) of acute flaps ($P < 0.05$). These data suggested that DT diaphorase activity was significantly enhanced after preconditioning as a bipedicle flap and the enzyme activity correlated with increased survival of the preconditioned flaps ($P < 0.05$).

To explore the temporal relationship between enhanced DT diaphorase activity and the duration of the preconditioned process in the bipedicle flaps, the distal attachment was divided at various days after the flaps were created. These data in Table 3 show that maximal nonstimulated DT diaphorase activity was greatest at 4 days. This DT diaphorase activity was increased 2-fold compared to the proximal ends of acute flaps and was the highest DT diaphorase activity recorded in this study. In the distal ends, there was a 14-fold increase in DT diaphorase activity ($P < 0.05$). Thereafter, baseline DT diaphorase activity decreased throughout the preconditioned flap, but remained higher than enzyme activity in acute flaps. Unfortunately, bipedicle skin flaps did not survive if the distal attachment was divided after 4 days of preconditioning.

To determine if there was a temporal relationship between increased DT diaphorase activity and increased steady-state levels of the message for DT, skin samples were obtained from the bipedicle skin flap when the enzyme activity was the highest. The Northern blot analysis shows that the steady-state levels of DT mRNA increased in the bipedicle flap at 4 days and correlated with enhanced enzyme function (Fig. 2). A ribosomal RNA control was used in these experiments to control for loading or transfer differences among samples. Equivalent amounts of total RNA from all samples were analyzed.

DISCUSSION

This study provides the first evidence that flavoproteins may participate in protection from oxidant stress.

TABLE 3

DT Diaphorase Activity (ΔAbs/min/100 g) in Bipedicle Skin Flaps That Were Preconditioned for 1, 3, 4, and 7 Days, the Distal Attachment Divided to Produce Ischemia, and then Assayed 24 Hours Later

Days of preconditioning	Proximal			Distal		
	Baseline	Tween	Dicumarol	Baseline	Tween	Dicumarol
1	2.5 ± 0.41	3.3 ± 0.49	0.033 ± 0.007	0.083 ± 0.024*	0.42 ± 0.175	0.001 ± 0.001
3	3.13 ± 0.37	3.95 ± 0.57	0.044 ± 0.01	0.283 ± 0.11*	0.517 ± 0.10	0.003 ± 0.002
4	4.60 ± 0.48	6.7 ± 0.68	0.058 ± 0.008	0.597 ± 0.11*	1.11 ± 0.15	0.005 ± 0.002
7	2.29 ± 0.45	3.8 ± 0.95	0.045 ± 0.015	0.234 ± 0.03*	0.874 ± 0.18	0.008 ± 0.004

* $P < 0.05$ compared to the proximal end using paired Student t tests and $N = 5$.

This is an attractive hypothesis since these ubiquitous proteins readily transfer free electrons formed from free radicals during ischemia using quinones as the intermediary substrate. Our data suggest that the enzyme remains active despite progressive ischemia and may be particularly effective as the intracellular milieu becomes unfavorable with progressive ischemia. The enzyme, DT diaphorase, is a flavoprotein with a M_r 55,000 which reduces quinones to the stable hydroquinone [12]. There is evidence that the naturally occurring ubiquinone reduces cellular damage from oxidant damage following ischemia–reperfusion after acute myocardial infarction [13].

The regulatory mechanism which governs DT diaphorase activity in skin ischemia is unknown. The enzyme's function has been reported to detoxify quinones formed from xenobiotic compounds found in tissue. While preliminary experiments have noted increased DT diaphorase enzyme activity in human keratinocytes after exposure to hydrogen peroxide (unpublished), previous investigators have shown elevated DT diaphorase activity in keratinocytes [9] and hepatocytes [14]. There is an increase in DT diaphorase activity in response to treatment with known carcinogens, such as 3-methylcholanthrene (3MC). Other investigators have shown that rats treated with 3MC express elevated levels of mRNA for DT in hepatocytes [15].

The increase in DT diaphorase mRNA and activity during early flap ischemia suggests that there is either increased gene expression or stabilization of message resulting in upregulation of DT diaphorase activity in the wound. These data supports the notion that increased enzyme substrates or increases in local cofactors are not solely responsible for enhanced enzyme activity. Increased DT diaphorase activity occurs in the proximal end of the skin flap where the tissue will survive.

The preconditioning effect may represent a combination of reduced intracellular oxidant activity [16], more effective glucose utilization [8], persistence of regional blood flow [7], or protection of endothelial cells. Our data supports the notion that other factors are involved in preconditioning since maximal DT diaphorase activity occurred at 4 days when there was no increased skin flap survival.

The utility of flavoproteins in treating ischemia remains speculative, although the therapeutic benefit associated with the two-electron transfer of quiones formed from free radicals may be significant. Some flavoproteins, such as riboflavin, present in the diet may potentiate antioxidants defences. Our preliminary observations with the flavoprotein, DT diaphorase, may possibly provide new thereapetic modalities for prevention of oxidant injury in tissue.

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